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THE CHANGING PATTERNS OF MOTOR INNERVATION IN THE DEVELOPING LIMB

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C O N T E N T S

SUMMARY

1. A technique using the retrograde axonal transport of horseradish peroxidase is developed and evaluated for use in determining which ventral horn cells are innervating various regions of the developing hind limb bud in Xenopus laevis.

2. An anatomical description of horseradish peroxidase labelled ventral horn cells at early stages of development is given. Particular attention is given to position, orientation and dendritic development.

3. The stages at which ventral horn cell innervation to proximal and distal limb regions first begins, are determined.

4. A limited study of the projection patterns of the ventral horn cells to the hind limb in the juvenile is made.

5. A study of the projection patterns of the ventral horn cells to the hind limb of the larva is made in successive stages from the stage of earliest motor innervation to the juvenile.

6. These studies indicate that at least the majority of nerve fibres probably are guided into specific limb regions though not necessarily the regions appropriate to the adult projection pattern. The form that this guidance might take is discussed.

7. Certain major changes in the larval projection patterns occur abruptly shortly before limb muscle function appears. These are analysed experimentally and shown to result from the death of ventral horn cells innervating limb regions inappropriate to the adult projection pattern.

8. The onset of this phase of cell death is related to developmental events in the limb, and possible mechanisms giving rise to selective cell death are discussed.

List of Abbreviations

Ach	Acetylcholine
AER	Apical ectodermal ridge
AP	Antero posterior
DAB	Diaminobenzidene (tetrahydrochloride)
DM-VL	Dorso medial - ventro lateral
DNA	Deoxyribonucleic acid
EDTA	Ethylene-diamine-tetra-acetic acid
EM	Electron microscope
H and E	Haematoxylin and eosin
HRP	Horseradish peroxidase
MEPP	Miniature end plate potential
NMJ	Neuromuscular junction
pH	Negative $\log_{10}$ hydrogen ion concentration in n moles/litre.
PZ	Progress zone
VHC	Ventral horn cell
w/v	Weight per volume
ZPA	Zone of polarising activity

ALA
ALT
Plantar
PMT
Distal
Mixed

See fig. 1.1.

FIGURES

TABLES

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DECLARATION

I declare that this thesis has been composed by myself and that the work described in it is my own.

PREFACE

The adult limb musculature is innervated* in an ordered and predictable fashion by the moto neurones of the ventral horn. Early in their development the primitive neurones, and the cells that will form the muscles, are unconnected and separated by a distance very large in relation to their size. The problem is, in what manner do the axons of the neurones find their way into the limb in such a way as to give always the same pattern of connections?

This thesis is a continuation of the large amount of work which has already been directed towards the problem. The approach taken here will be to examine the early patterns of innervation to the developing limb bud in the hope of deducing the mechanisms controlling the formation of the ordered nerve muscle relationships of the adult. Xenopus laevis is used for its convenience and in the hope that principles derived from this animal may be applicable to man.

The point of contact between the neurone and the muscle cell is within the limb. Many of the mechanisms sought for may largely depend on factors in the limb. Therefore, a great deal of emphasis is placed on developmental events there and it is appropriate for the thesis to begin at this point.

* See footnote p.4.

CHAPTER 1

INTRODUCTION

1.1 Developmental Events in the Limb

1.1.1 Development of the limb

The limb first becomes distinguishable at Stage 45* as a condensation of mesenchyme in the caudo-ventral aspect of the abdominal wall (Newth, 1967). By Stage 49 it is a distinct swelling and the epidermis over the apex has become thickened to form the apical ectodermal cap. Proliferation of the mesenchyme results in growth and elongation through a series of characteristic shapes (Fig. 1.1). It is roughly spherical at Stage 50 and pointed at Stage 51. By Stage 52 it has a clear ankle indentation and by Stage 53 the foot paddle is apparent. By Stage 54 the toes are differentiating. No regional differentiation of the mesenchyme is seen until Stage 52 when condensation giving rise to the femur begins.

Myotube formation begins in the proximal part of the thigh in Stage 53 (Newth, 1967) and simultaneously, proximal muscle becomes directly electrically excitable (Hughes and Prestige, 1967) (Fig. 1.2). In Stage 54 early striations are visible proximally and rapid muscle fibre formation enables identification of some individual thigh muscles, some of which have formed both their origins and insertions

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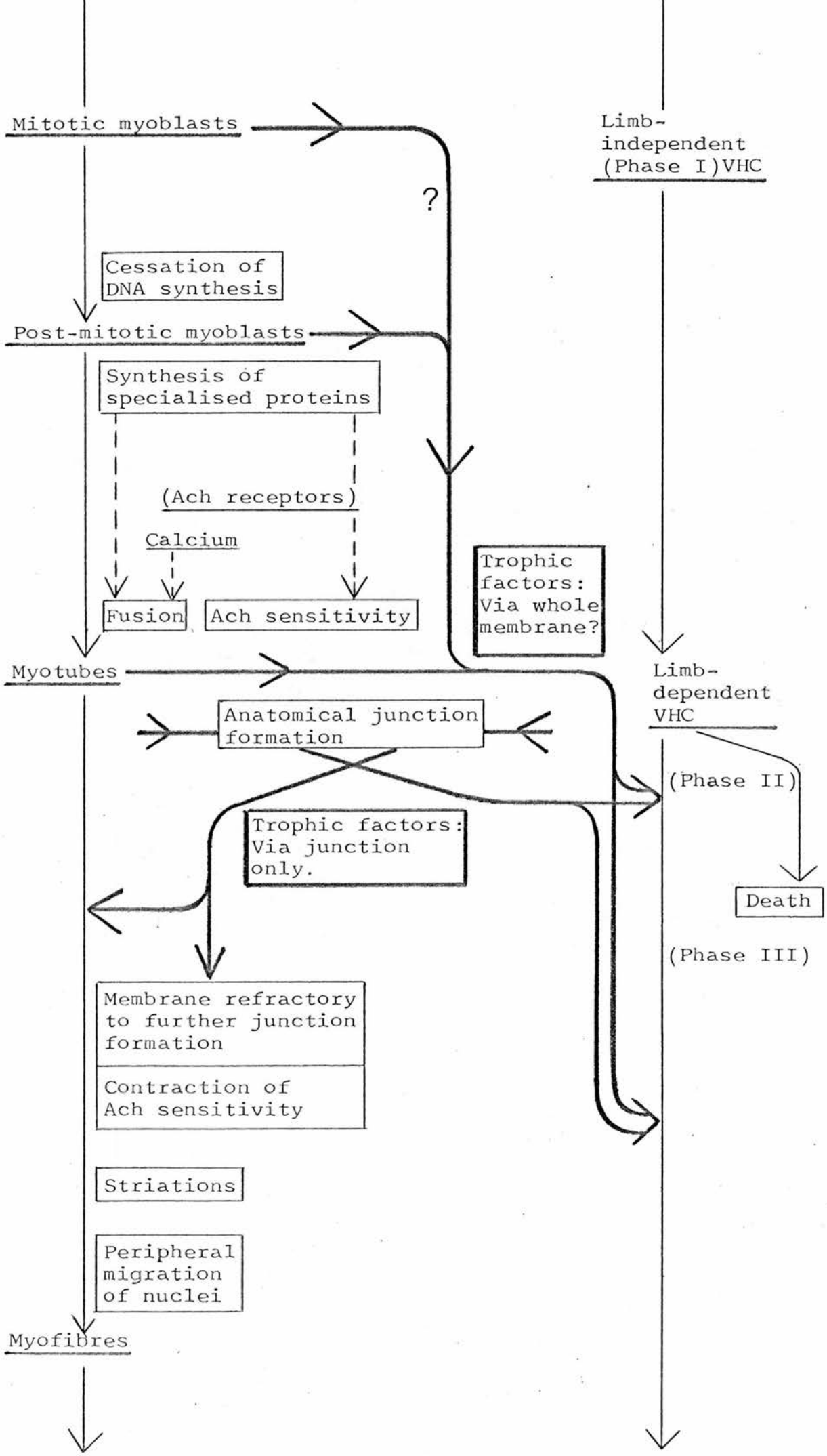
* All stages according to Nieuwkoop and Faber, 1967.

Fig. 1.2

The order of developmental events in the myogenic cells and the ventral horn cells and the probable trophic interactions between the two populations. All myogenic events shown except myofibre formation take place within one stage. In the thigh these occur in Stage 53. Myofibres appear in Stage 54. More distal limb regions reach these stages later. Ventral horn cells reach limb dependence from late Stage 52 onwards. Light boxes: developmental events. Heavy boxes: route of trophic factors. Light arrows: time direction. Heavy arrows: trophic actions. Dashed arrows: known causality (other than via trophic factors).
VHC = ventral horn cell.

See page 10 for definition of Phases I, II, III.


Mitotic myoblasts
?
Limb-independent (Phase I) VHC
Cessation of DNA synthesis
Post-mitotic myoblasts
Synthesis of specialised proteins
(Ach receptors)
Calcium
Fusion
Ach sensitivity
Trophic factors: Via whole membrane?
Myotubes
Limb-dependent VHC
Anatomical junction formation
(Phase II)
Trophic factors: Via junction only.
Death
(Phase III)
Membrane refractory to further junction formation
Contraction of Ach sensitivity
Striations
Peripheral migration of nuclei
Myofibres

(Newth, 1967). Simultaneously the first movements at proximal joints begin, both spontaneously and on stimulation of the spinal nerves (Hughes and Prestige, 1967). More distal parts of the limb attain these stages of development at successively later times such that there is a difference in time of development between proximal thigh and foot of two or three stages.

The time gradient results from the manner in which the parts of the limb are evolved. The most proximal parts of the limb are determined first while progressively more distal parts are determined and generated in the apical region of the growing limb bud at successively later stages (Tschumi, 1957). The function of the apical region requires the presence of an intact apical ectodermal cap. Its removal results in cessation of determination of regions more distal to those already determined giving rise to a mature limb in which proximal structures already determined are normal whilst more distal structures are lacking (Tschumi, 1957). In the chick, whose limb development conforms to the same principles (Saunders, 1948), the apical region has been found to consist of at least 95% dividing cells (Summerbell and Lewis cited by Wolpert et al., 1975). Confirmation that local cellular proliferation and not distal migration of proximally generated cells is responsible for the apical generation of more distal regions comes from experiments involving the construction of limb bud chimeras (Cairns, 1965; Amprino, 1965).

Further work on the chick has led to a partial understanding of the mechanisms responsible for the

determination of the limb axes (Fig. 1.3). A region at the postero-medial junction of the limb bud with the body wall called the zone of polarising activity (ZPA) has been shown to determine the antero-posterior (AP) axis (Saunders and Gasseling, 1968; Saunders, 1972; Summerbell, 1974a; Tickle et al., 1975). It acts on a region of the apical mesoderm termed the progress zone (PZ) (Summerbell et al., 1973) perhaps via a diffusing substance since a ZPA transplanted totally outside the limb bud but near its base can influence the AP axis (Tickle et al., 1975). However no activity can be transferred via gel or tissue placed in contact with ZPA tissue for twelve hours in vitro (Saunders and Gasseling, 1968).

The progress zone is a recent concept used to explain the generation of the proximo-distal axis (Summerbell et al., 1973). It is estimated to be 230 μm ± 70 μm thick (Wolpert et al., 1975). According to the concept, positional information (Wolpert, 1969,1971) is imparted concerning the proximo-distal axis by the amount of time cells or their progenitors have been in the PZ at the moment they leave it. As cells are continually forced out of the PZ by proliferation within the zone they acquire graded proximo-distal positional information. Predictions based on this model are approximately borne out in transplantation experiments (Wolpert et al., 1975).

The role of the apical ectodermal ridge (AER), the equivalent of the apical ectodermal cap in Xenopus, is that of a permissive agent upon which the continued function of the PZ depends (Rubin and Saunders, 1972; Summerbell, 1974b).

The AER does not itself impart positional information (Rubin and Saunders, 1972). In turn the AER depends for its function on the presence of the ZPA (Saunders and Gasseling, 1968; Summerbell, 1974a) which, however, acts indirectly through the apical mesenchyme (Saunders, 1972).

The mechanisms determining the dorso-ventral axis are unknown. Saunders (1972) suggests that the axis is fixed at a stage well before the limb bud begins to form.

1.1.2 Development of Muscle

No work has been done on muscle fibre development in Xenopus. Almost all the knowledge in this field is derived from tissue culture work for which various sources of muscle cells have been used, most frequently axial in origin. In addition all the sources used by the authors cited had been innervated* already, thus it is not known whether all of the changes described below can occur in the absence of at least temporary nervous contact either of the cells being investigated or their progenitors. This is particularly relevant in view of the finding that muscle derived from previously innervated tissue has certain differences to that derived from previously uninnervated tissue (Yntema, 1959). Extrapolation of much of the following information to Xenopus limb muscle development in vivo must therefore be made with caution (Fig. 1.2).

The fusion of myoblasts into myotubes is well described (e.g. Stockdale and Holtzer, 1961; Konigsberg, 1963; Cairns, 1965; Okazaki and Holtzer, 1966; Rash and Fambrough, 1973; Yaffe, 1973). It is preceded and accompanied

* The meaning of the term "innervation" and its derivatives as used in this thesis is: the presence of nerve endings. It does not necessarily imply any anatomical or functional state of the endings such as the presence of neuromuscular junctions. Such states cannot be distinguished by the techniques described in this thesis.

by profound changes in the myoblasts including the cessation of DNA synthesis and subsequent synthesis of specialized proteins without which fusion cannot proceed (Stockdale and Holtzer, 1961; Okazaki and Holtzer, 1966; Bischoff and Holtzer, 1970; Easton and Reich, 1972). The timing of the final cell division is related to the absolute age of the myoblasts and a certain number of cell divisions are required before the final division (Yaffe, 1973). Coincidental with fusion, acetylcholine (Ach) receptors, which have been shown to be a specialized protein using an α- bungarotoxin binding technique (Miledi, Molinoff and Potter, 1971), appear in large numbers over the whole myotube membrane though a few may appear on post-mitotic myoblasts immediately prior to fusion (Sytkowski et al., 1973). This is corroborated by the finding of marked sensitivity to Ach over the whole membrane of uninnervated myotubes in vitro (Harris et al., 1973) and corroborates the finding of the appearance and rapid rise in specific activity of Ach receptor protein in vivo in chick limb buds at the time of myotube appearance (Giacobini et al., 1973). However Ach sensitivity is not an effect of fusion as it will still rise to the usual levels if fusion is prevented by the removal of calcium ions from the culture medium (Fambrough and Rash, 1971a; Patterson and Prives, 1973). Ach sensitivity plays no part in fusion itself (Fambrough and Rash, 1971a).

High concentration clusters of Ach receptors identified by autoradiographic location of tritiated α-neurotoxin which binds selectively to Ach receptors, develop at random sites

on the membranes of uninnervated myotubes (Vogel et al., 1972; Sytkowski et al., 1973; Cohen and Fischbach, 1973) coincidentally with the appearance of peaks of Ach sensitivity on the membranes (Fischbach and Cohen, 1973) and average about twenty clusters per myotube (Sytkowski et al., 1973). The suggestion has been made that such sites may form part of the recognition system allowing growing axons to synapse at that point (Sytkowski et al., 1973; Fischbach and Cohen, 1973). However, the time required for the formation of the clusters after myotube formation was about four days whereas in vivo, anatomical junction formation begins shortly after myotube formation (Hirano, 1967; Terravainen, 1968; Kelly and Zacks, 1969; Bennett and Pettigrew, 1974a).

1.2 Developmental Events in the Spinal Cord

1.2.1 Development of the ventral horn

The ventral horn is first apparent histologically at Stage 50 as a circumferentially wide, thin column of tangentially oriented neuroblasts at the lateral periphery of the spinal grey matter in the lumbar region (Hughes, 1961; Prestige, 1967,1970). Accumulation of further neuroblasts results in expansion of the column radially. This combined with dorsal growth of the spinal cord gives rise to the typical long, thin ventral horn of the adult which projects radially from the grey matter into the white matter ventro-laterally as seen in transverse section. Medially, there is a quite separate column of motor cells, the primary motor neurones which innervate the axial

musculature and do not innervate the limb in the anurans (Silver, 1942; Hughes and Tschumi, 1960; Prestige and Wilson, 1972; Cruce, 1974; Letinsky, 1974) (Fig. 1.3).

A recent autoradiographic study has shown that the neuroblasts of the ventral horn arise from the ventricular layer and migrate to the lateral side of their predecessors resulting in the oldest neuroblasts being medial to the youngest (Prestige, 1973)(Fig. 1.3). This occurs also in the chick (Fujita, 1964). The apparent discrepancy of this work with other work in the chick (Langman and Haden, 1970) and rat (Nornes and Das, 1974) which superficially suggests a latero-medial order of laying down arises because the latter workers are dealing with the whole anterior horn and do not describe events in the ventrolateral column, corresponding to the ventral horn, alone.

Corroborating evidence for a medio-lateral order of laying down is presented in this thesis.

The time between the final division giving rise to the neuroblast and its arrival in the ventral horn is about four days or a stage and a half (Prestige, 1973). The last neuroblasts are generated by Stage 53 and have entered the ventral horn by the end of Stage 54 (Prestige, 1973). Previous evidence giving rise to the suggestion that new neuroblasts enter the ventral horn throughout larval life thus resulting in massive cell turnover (Hughes, 1961) was based on assumptions and estimations that cannot be considered reliable (Kollros, 1968). No evidence of this scale of turnover is presented by workers in the chick

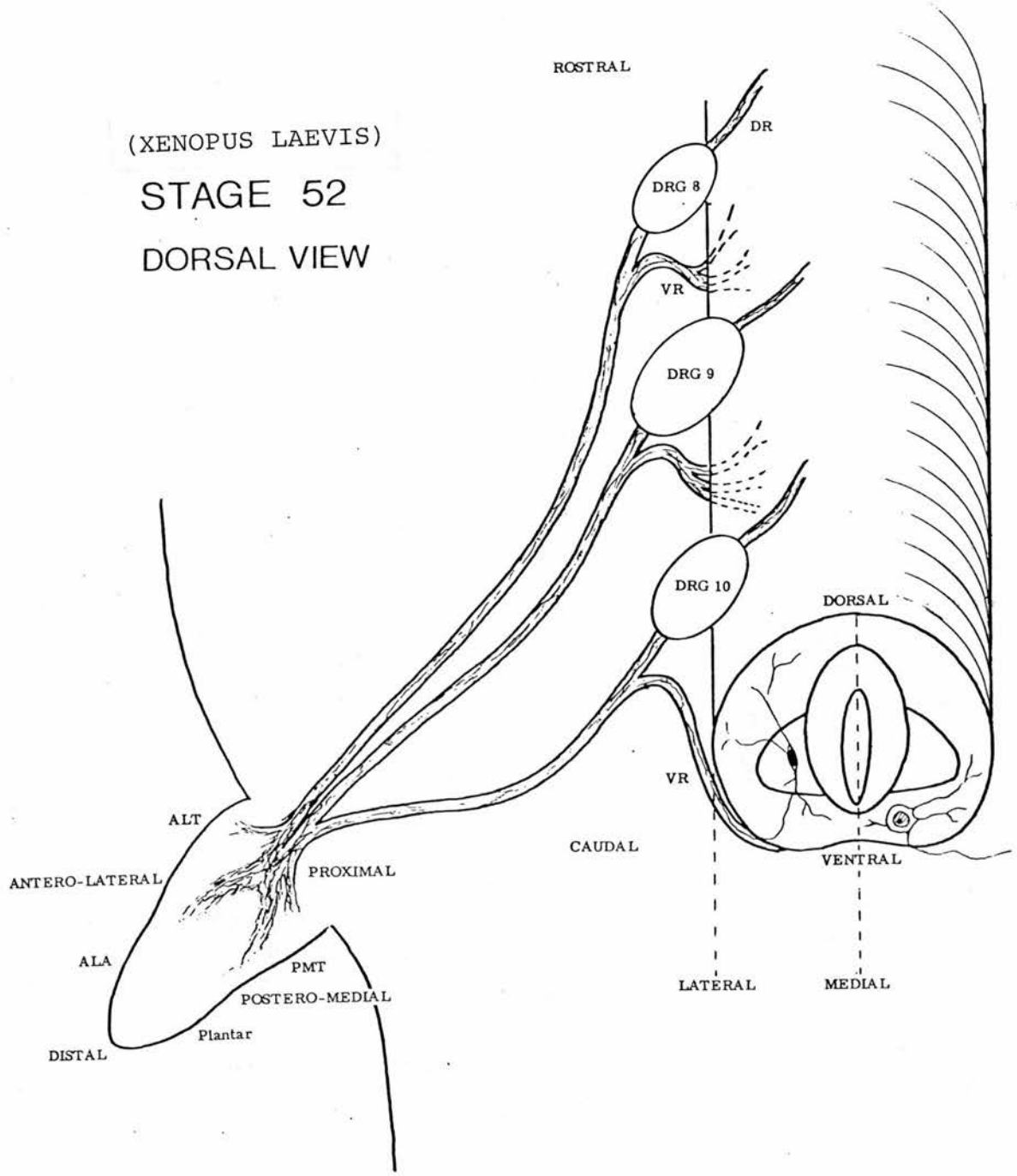


Fig. 1.3

Semi-Schematic diagram to show axes and layout of the spinal cord, spinal ganglia and nerves and relationship of the nerves to the limb bud in a Stage 52 animal. Nerve trunks approaching and in the limb bud are accurately drawn from an actual single H and E stained specimen with a typical nerve trunk arrangement. A typical ventral horn cell is shown on the left side of the cord and a primary motor neurone on the right. Dorsal root ganglion numbering is after Gaupp, 1899. DRG = dorsal root ganglion. DR = dorsal root. VR = ventral root. Limb abbreviations as in Fig. 1.1. H and E =haematoxylin and eosin.

(Hamburger, 1958, 1975) or mouse (Harris, 1969).

The difference in age between the oldest or most medial neurones and the youngest or most lateral neurones is about three stages (Prestige, 1973).

The rostral end of the ventral horn (Fig. 1.3) is more advanced developmentally than the caudal end (Hughes, 1968; Prestige, 1970,1973). This phenomenon is seen in other amphibia (Coghill, 1933) and higher vertebrates including chick (Hamburger, 1948; Fujita, 1964) and rat (Kingsbury, 1926; Nornes and Das, 1974). In Xenopus the difference is just under a stage, thus the whole length of the ventral horn is present by Stage 51 (Prestige, 1973).

1.2.2 Development of ventral horn cells

The youngest neuroblasts are small and densely packed with oval nuclei about 10 μm long (Hughes, 1961) filling nearly the whole perikaryon, and containing a small nucleolus. It is generally agreed that the nuclei are larger and paler staining than the adjacent mantle layer nuclei in all species studied (Hughes, 1959; Langman and Haden, 1970; Romanes, 1942; Hughes and Tschumi, 1958; Hughes, 1961; Prestige, 1967, 1970). However some workers have described the very earliest neuroblasts as being virtually indistinguishable from mantle nuclei (Barron, 1943, 1946; Beaudoin, 1955; Pollack, 1969) giving rise to problems of correct identification (Hughes, 1961; Prestige, 1973). By Stage 52 in Xenopus the cells are bipolar with small caps of cytoplasm

at either end and by Stage 55 a layer of cytoplasm is visible all the way around the nucleus which itself has increased considerably in size (Hughes, 1961; Prestige, 1970). A more detailed description of ventral horn cells development has been given for Rana pipiens (Beaudoin, 1955) which closely fits the description for Xenopus. This study contains the only description of dendritic development at early stages of the ventral horn but the description is brief and vague. Barron (1943,1946) describes dendritic growth in developing sheep and chick spinal cord but it is not clear that his primary neuroblasts from which the dendrites extend are not axial moto-neurones.

Descriptions of adult anuran ventral horn cells have been given for R. pipiens (Silver, 1942) and R. temporaria (Kennard, 1959). In general these are similar though the ventral horn cells of R. pipiens are larger (120 μm) than R. temporaria (50 - 70 μm).

The cells are spindle shaped or polygonal and multipolar, the dendrites tending to extend from the dorsal and ventral poles. The initially broad based dendrites branch profusely into the sub-pial zone of the lateral funiculus, the dorsal neuropil within and below the dorsal horn, the neuropil ventral to the ventral horn and to the opposite side of the cord via the ventral commissure.

1.2.3 Ventral horn cell numbers

The number of neurones in the ventral horn increases rapidly up to Stage 53 when a maximum of about 6000 is

reached. Thereafter, many cells degenerate (Nieuwkoop and Faber, 1956; Hughes, 1961) resulting in a fall in number to about 1500 in the adult, the peak rate of fall being in Stages 54 and 55 and largely complete by Stage 57 (Prestige, 1967). There is a small temporal overlap between the last of the incoming neuroblasts and the first of the degenerations.

According to their response to amputation of the ipsilateral limb, three classes of ventral horn cells can be described. They have been labelled Phase I, Phase II and Phase III (Prestige, 1967, 1970). Phase I show no response and are apparently limb-independent. Phase II cells degenerate almost immediately and Phase III degenerate after a delay whose length increases with the maturity of the cell. The end of Phase I marks a major qualitative change in the cell from limb independent to limb dependent. The exact significance of Phase II cells is still obscure (Prestige - personal comm.) In the normal animal all cells are Phase I up to Stage 52. After this the number of Phase II cells closely corresponds with the number of degenerations occurring up to about Stage 56 in unoperated animals. Phase III cells increase gradually in number from Stage 54 but some of these degenerate between Stages 56 to 59 (Prestige, 1970).

No cause or purpose for degenerating cells has been shown. The suggestion that the cells that degenerate have failed to innervate the limb has been recently disproved in an unpublished experiment which shows that all ventral

horn cells grow an axon into the limb (Prestige and Wilson - personal communication). This evidence was obtained by making serial fibre counts of the ninth ventral root and spinal nerve as far as the base of the limb. Only a small fall in fibre count consistent with the youngest fibres having grown only part of the way at the time of fixation, is seen with progressively more distal counts. This work supports the indirect conclusions reached earlier (Lamb, 1974; Prestige and Wilson, 1974) and corroborates and extends previous work showing that all ventral horn cells grow an axon through the ventral root within a day or two of appearing in the ventral horn (Prestige and Wilson, 1972, 1974).

Evidence is presented in this thesis to show that at least some degenerating cells have innervated regions of the limb inappropriate to the region of the ventral horn in which the cell bodies are sited.

1.2.4 Timing of the earliest motor innervation to the limb bud

Although the time at which ventral horn cell axons first innervate the limb bud is important no direct attempts have previously been made to determine this. Nerve fibres have been described within the limb buds of Stage 49 in Xenopus (Hughes and Tschumi, 1958) but no information has been given regarding the origin of these fibres. In Rana pipiens, nerve fibres have been described in Stage L4 limb buds (equivalent to Stage 50 in Xenopus)(Taylor, 1943). Some of these were shown to have traversed the ventral

root and it was argued that they were ventral horn fibres. However no direct test of this assertion was made. The timing of the earliest ventral horn innervation to the limb bud has been determined directly in this thesis. Some of the results leading to this determination have been published (Lamb, 1974). Information is also presented here regarding the timing of ventral horn innervation to individual regions of the limb bud.

1.2.5 Ventral horn projection into the adult limb

Studies on Xenopus involving amputation (Hughes, 1968; Prestige - personal communication) and spinal nerve stimulation (Hughes and Prestige, 1967) conform with the generalization arising from the work of Kaiser, 1891 (cited by Sprague, 1946) that proximal muscle is supplied by more rostral ventral horn cells than distal muscle. Further generalizations about vertebrate moto-neurone organization, namely that embryologically dorsal musculature is supplied by lateral ventral horn cells and embryologically ventral musculature by medial ventral horn cells (Bikeles, 1905 cited by Sprague 1946) or that extensor ventral horn cells tend to be caudal to flexor (Silver, 1942) have been so far neither confirmed nor denied in Xenopus.

The first detailed studies of lumbar ventral horn cell projection to be carried out in any animal were on the cat (Elliott, 1944; Romanes, 1951). In addition to finding that similar generalizations to those outlined above can be made about the mammal, this study made it clear that the ventral horn is subdivided into many regions

corresponding to different muscle groups. An equally detailed study on Ambystoma showed only a vague somatotopic subdivision of motor neurones, neurones for different muscle groups being scattered almost at random. However, in Ambystoma there are no demarcated motor columns and the cord appears primitive in organization compared to all the higher vertebrates including the anura (Piatt, 1939; Strauss, 1946; Székely and Czéh, 1967). Confirmation that the anuran motor column is organised in a similar way to the cat has come very recently in a detailed study on R. catesbeiana (Cruce, 1974). The similarity of organization between the two species is remarkable in view of the wide phylogenetic separation.

A restricted study of the juvenile Xenopus ventral horn projection to certain regions of the hind limb has been carried out in this thesis.

1.2.6 Ventral horn projection into the larval limb

Some attempts have been made to define indirectly the patterns of ventral horn cell projection to the developing limb bud in Xenopus (Hughes, 1968) but only in the broadest of terms. Stimulation of the spinal cord rostral to the lumbar region (Fig. 1.3) resulted in generalized contractions of the limb bud at Stage 54 while later on in development discrete movements occurred. Stimulation of the tenth spinal nerve (Fig. 1.3) resulted in different movements as the animal developed from Stage 54 onwards. Hughes interpreted these findings as showing an initially random innervation becoming organized with development. However

the presence of other uncontrollable variables make these observations of little value in this respect. However, a recent histochemical study of the developing chick limb bud gives some support to this idea (Giacobini et al., 1973). Evidence was found of a fall of the specific activity of choline acetyl transferase, an indicator of cholinergic nerve terminal presence, during the period when cell degenerations in the chick ventral horn are occurring (Hamburger, 1958,1975). It is possible that this represents an absolute fall in terminal numbers rather than simply a relative fall resulting from muscle growth as the authors suggest. An absolute fall would indicate that not all terminals are permanent.

An anatomical approach was taken by Taylor (1943) who studied histologically the distribution of the earliest nerve fibres in the limb bud of Rana pipiens. He concluded that motor fibres are committed to the appropriate part of the limb from the moment of entry into the limb. However there are objections in this work also. The course of individual immature fibres cannot be ascertained by light microscopy, nor can observation of the peripheral fibre tell from where it originated.

Hughes (1968) observed in developing Eleutherodactylus hind limb buds that the maturity of the ventral horn cells innervating either proximally or distally was related to the maturity of the muscle being innervated. From this he proposed that ordered innervation of the limb results from the coincidental timing of development of limb regions and corresponding ventral horn cells. The results of the present

work disprove this hypothesis.

In this thesis, an analysis of the patterns of motor innervation to the developing hind limb bud from the time of earliest innervation to the juvenile has been carried out and examined in relation to the possible mechanisms directing the formation of connections appropriate to the adult pattern. For this reason, a brief discussion of the main theoretical proposals in this respect will be presented after the following section dealing with the development of the neuromuscular junction.

1.3 Development of the Neuromuscular junction

There have been few studies of neuromuscular junction (NMJ) formation in anurans. The development of the neural side of neuromuscular transmission has been studied in R. catesbeiana by forcing early ventral horn cells to innervate mature tail muscle (Letinsky, 1974). Ventral horn cells equivalent to Phase I in Xenopus were found to be capable of initiating muscle contraction.

Anatomical descriptions of NMJ formation have been made for limb muscle of chicks (Hirano, 1967; Bennett and Pettigrew, 1974a) axial muscle of rat (Terravainen, 1968; Kelly and Zacks, 1969; Bennett and Pettigrew, 1974a) and in vitro (James and Tresman, 1969; Robbins and Yonezawa, 1971). In principle all these descriptions agree. The first anatomical indication of NMJ formation appears only after myotube formation (Fig. 1.2) as sarcolemmal thickening and early basement membrane formation. Prior to this stage, however, there is no uniform agreement about the

relationship of axon endings to the myoblasts. Descriptions vary from total enclosure of the endings in Schwann cell cytoplasm (Terravainen, 1968) to partial engulfment of endings by the myoblast membrane (James and Tresman, 1969). However, technical objections arise in the latter making this description less reliable. Firstly the myoblasts were obtained by trypsin dispersal of muscle that had already been innervated and contained myotubes; secondly no checks were made to ensure that the muscle cells described were mononuclear, the only criterion for calling them myoblasts being that they contained no myofibrils though early myotubes also may have no myofibrils (Okazaki and Holtzer, 1966; Fischman, 1967). It is probable that endings lie partially enclosed by Schwann cells but also in direct but not close contact with myoblasts (Hirano, 1967; Kelly and Zachs, 1969).

It is probable that only one post-synaptic site develops on each myotube (Bennett and Pettigrew, 1974a) though initially this site may be innervated by more than one ending (Hirano, 1967; Terravainen, 1968; Bennett and Pettigrew, 1974a) which may be derived from more than one moto-neurone (Shimada et al., 1969; Redfern, 1970; Bagust et al., 1973). Polyneuronal innervation was found in early stages of innervation of mature tail muscle by immature ventral horn cells in R. catesbeiana (Letinsky, 1974).

Junction formation results in the muscle membrane rapidly becoming refractory to further junction formation (Fig. 1.2) for a finite distance which may include the whole

membrane depending on the type of nerve (Bennett and Pettigrew, 1974a). A permanent membrane change is involved as the process cannot be completely reversed by denervation. Reinnervation preferentially takes place at the old post-synaptic site or on new muscle membrane added by further fusion during the period of denervation (Bennett and Pettigrew, 1974b). Coincidental with the development of membrane refractoriness, but more slowly, the area of Ach sensitivity contracts to the region of the end plate (Diamond and Miledi, 1962; Kano and Shimada, 1971). This process is completely reversible by denervation (Miledi, 1960a; Axellson and Thesleff, 1969) botulinum poisoning (Thesleff, 1960) and nerve block by local anaesthetics (Lomo and Rosenthal, 1972). But this can be prevented by inhibition of new protein synthesis (Fambrough, 1970) or direct electrical stimulation of the muscle both in vivo (Lomo and Rosenthal, 1972) and in vitro (Cohen and Fischbach, 1973). Ach itself does not control the extent of Ach sensitivity since no restriction of sensitivity of denervated muscle occurs by bathing it in Ach solution (Miledi, 1960). Also sensitivity restriction occurring during reinnervation begins after reinnervation but before neuromuscular transmission of impulses when only low frequency MEPPs are occurring (Miledi, 1960b)and in addition it is not prevented by preventing Ach release (Steinbach et al., 1973). The causal relationship, if any between Ach receptors and membrane refractoriness and junction formation has not been determined, however, it has been shown that normal NMJ formation is prevented in

developing chick limb by curare, botulinum toxin and hemicholinium (Drachman, 1968) and α-neurotoxin (Giacobini et al., 1973) though another study showed that NMJs in axial musculature could develop in the presence of curare (Cohen, 1972).

Furthermore, in a recent study in adult rats where soleus muscle fibres were hyperinnervated by foreign fibular nerve fibres in addition to the normal tibial nerve fibres, it was shown that the foreign synapses developed at a new point on the muscle membrane despite the suppression of extra junctional Ach sensitivity increase by electrical stimulation of the muscle (Frank et al., 1975).

1.4 Theories of Innervation

The theoretical proposals can be divided into two categories many of which are derived from work on other systems. Certain proposals cannot apply to developing limb motor innervation other than at a minor level owing to the known existence of a non-random ventral horn projection in the adult They are the resonance principle (Weiss, 1928) and its descendents including the modulation theories (Weiss, 1936,1942; Kolross, 1943; Sperry, 1941, 1950; Jacobson and Baker,1968, 1969). They will not be discussed.

The two categories are:

1. Those that postulate that initial innervation takes place at random with subsequent reorganisation into the adult pattern.

2. Those that postulate that fibres are constrained to follow prescribed pathways to prescribed areas or post-

synaptic sites.

Chief amongst the category one proposals are:

1. The redundancy hypothesis: An over production of neurones ensures that sufficient of the randomly innervating neurones find an appropriate post-synaptic site. All those not succeeding die (Hamburger and Levi Montalcini, 1949; Race, 1961; Race and Terry, 1965; Hughes, 1965; Hughes and Prestige, 1967; Hughes, 1968; Kollros, 1968; Prestige, 1970; Hamburger, 1975).

2. The branching hypothesis: Each neurone sends branches to all areas with only the appropriate branches surviving (Hughes, 1964a,b, 1965, 1968). However, recent unpublished evidence obtained from Xenopus larvae of early limb bud stages suggest that branching is at most a minor feature at least down to the level of the larger nerve branches within the limb (Prestige and Wilson - personal communication).

3. The moving endings hypothesis: Fibres initially innervate at random and then continue moving through the tissue until an appropriate site is found. This has arisen chiefly from work on the developing retino-tectal system of Xenopus where it plays an important part (Gaze et al., 1972, 1974). However no evidence for or against has been produced for the limb.

Category two proposals place the selective emphasis on the pathway giving the intercellular junction a minor role in the formation of appropriate connections:

1. Mechanical guidance

Mechanical factors probably play some role in the direction of nerve fibre growth. Growing neurites in vitro require a surface or interface upon which to grow (Harrison,

1910; Weiss, 1934). Fasciculation, the tendency for fibres to grow along the surface of other fibres, is probably an expression of this property. The direction of growth of neurites can be influenced by the orientation of the constituents of the material through which they are growing so that they tend to grow along lines of tension as for instance in stretched clotted plasma (Weiss, 1934) Whether similar forces act on growing neurites in vivo is not known though it has been suggested in developing Rana tadpoles as a means for guiding outgrowing ventral horn cell axons to the base of the limb (Taylor, 1943).

However, it has been shown that fibres can still reach their destination despite mechanical interference with normal pathways (Detwiler, 1936; Piatt, 1942; Hughes and Tschumi, 1958) or rerouting them via abnormal pathways (Arora, 1963; Hibbard, 1965, 1967).

2. Selective fasciculation

The earliest fibres make connection either by random searching or by pathway direction. These have been called pathfinder fibres in the past (Harrison, 1910; Weiss, 1941a; Barron, 1943; Taylor, 1943). Later fibres are guided by the pathfinders to the correct site (Weiss, 1941a). However pathfinder fibres have been shown to be unnecessary in the formation of normal nerve trunks (Piatt, 1942).

3. Chemospecific guidance

Fibres continually sample their immediate environment and detect differences enabling them to grow along specified pathways (Hamburger, 1962; Sperry, 1963; Attardi and Sperry, 1963). The seeking and apparent sampling behaviour

of growth cones has been well described (Speidel, 1941, 1942; Hughes, 1953; Sperry, 1963; Chalmley et al., 1973) and gives some support to this otherwise vaguely defined proposal.

4. Timing hypothesis

Axons and post-synaptic cells each belong to a temporarily ordered set. Only those axons and cells which match in maturity can connect. A variant of this proposes that only the set of axons is temporally graded, the earliest innervating the nearest post-synaptic cell and later ones being forced to further cells in order (Romanes, 1942; Jacobson, 1960; Hughes, 1968; Cruce, 1974). Time being one dimensional, such mechanisms can only operate in one dimension and can, at most, play only a minor role in two dimensional (e.g. retino-tectal) or three dimensional (e.g. ventral horn-limb) innervation (Prestige and Willshaw, 1975).

Various other theories of directed axonal growth along pathways have been proposed in the past which are now only of historical interest including stimulogenous fibrillation (Bok, 1915), electrical tropism (Ingvar, 1920; Marsh and Beams, 1946), neurobiotaxis (Ariens Kappers, 1921) and chemotropism (Child, 1921; Herrick, 1925; Cajal, 1929).

Various proposals have also been made concerning the mechanism by which axon endings and post-synaptic sites recognize each other as appropriate or inappropriate. The phenomena that these proposals refer to are not to be confused with the phenomena referred to in the previous two categories of proposals just discussed. Those two

categories were concerned with the means by which axon endings and post-synaptic sites are brought into apposition.

1. Matching labels hypothesis

Each pre-synaptic and each post-synaptic cell is given a unique label of unspecified type. Each pre-synaptic label has a post-synaptic counterpart which enables that pair of cells with those labels to recognise each other as appropriate. This model permits no connection between pairs of cells with non-matching labels but does not disallow the changing of labels to permit previously incompatible cells to become compatible (regulation). The labels themselves can be envisaged as being randomly mixed within each of the sets of cells or being organized so that the label property is graded across the cell sets (Sperry, 1943,1944,1945,1963; Meyer and Sperry, 1973). It has been postulated that gradients of this type may be derived from a system or systems imparting positional information to the cells (Wolpert, 1969,1971).

2. Connection plasticity hypothesis

Each fibre if acting individually, may synapse at any post-synaptic site. However this general recognition is modified in practice by the presence of other fibres (contextuality - Hunt and Jacobson, 1974) which results in the restriction of recognition to the appropriate site, appropriateness in this case being defined in terms of relative positions of cells within the arrays (Straznicky et al., 1971; Gaze and Keating, 1972; Hunt and Jacobson, 1974). A formal competition model based on this principle has recently been developed (Prestige and Willshaw, 1975).

1.5 Tolerance of the Selection Mechanism in Development

In order to obtain experimental information about the mechanisms of appropriate connection formation, some workers have forced heterotypic nervous innervation of various tissues to observe the tolerance of these mechanisms. With marked interspecies uniformity in all vertebrates tested, the range of nerve types that can form permanent functional NMJs in limb muscle during development is very limited. Neither sensory nerves in Rana pipiens (Taylor, 1944) nor axial moto-neurones in Ambystoma (Piatt, 1940, 1957; Székély, 1963) or Eleutherodactylus (Hughes, 1964a) or chick (Straznicky, 1963; Székély and Szentagothai, 1962; Straznicky, 1967) nor cranial nerves in Ambystoma (Piatt, 1956, 1957) or Eleutherodactylus (Hughes, 1964a) can do so.

In those experiments where the state of innervation was examined during the period of the developing innervation it was found that adequate innervation occurred (Hughes, 1964a; Straznicky, 1967) but junctions either did not form as in the chick (Straznicky, 1967) or were aborted after initial formation as in Eleutherodactylus (Hughes, 1964a). During the latter, a brief period of neuromotor transmission was reported, though it was not certain that axial moto-neurones had been responsible although the innervation came from a spinal nerve which normally does not supply the limb.

Permanent functional NMJs can form between limb and either of the limb moving regions of the spinal cord whether brachial or lumbar in Ambystoma (Piatt, 1956,1957; Székély, 1963), Eleutherodactylus (Hughes, 1962,1964a,b,

1968) and chick (Straznicky, 1963). In all cases where a limb is innervated by the heterotypic ventral horn, abnormalities of coordination occur. This probably reflects the imposition of the central organisation of the innervating region upon an unsuitable limb (Straznicky, 1963; Székély, 1963) rather than abnormalities of NMJ formation. However transplanted heterotypic limbs are less efficient at maintaining the ventral horn cell population number than transplanted homotypic limbs in Eleutherodactylus (Hughes, 1962) suggesting more fundamental differences between lumbar and brachial ventral horn cells. However this may reflect a difficulty that ventral horn cell axons have in following pathways in heterotypic limbs as has been shown in Ambystoma (Piatt, 1952, 1956, 1957) and may result in their becoming lost. The importance of this factor can be understood by considering two experiments which suggest that the neurones innervating proximal and distal limb muscle cannot be interchanged. In Ambystoma, if only moto-neurones which normally supply the distal part of the forelimb innervated the forelimb during development then movements were obtained only at distal joints (Detwiler, 1936).

Also in Ambystoma, another experiment involving spinal cord transplants at the time of spinal cord regional determination, produced similar results (Straznicky, Székély, 1967). However, in this, innervation also took place by moto-neurones determined as thoracic which may have acted to force the brachial limb moto-neurones to synapse distally, though they themselves were unable to

maintain permanent contact.

The mechanisms by which axon endings are accepted or rejected by a muscle fibre during development is quite unknown. Of relevance to this question is a series of studies of regenerating motor nerves in fish and tailed amphibians. In these animals, muscle fibres are poly-neuronally innervated in the adult resembling developing neuromotor systems of higher vertebrates. In this series of studies, synapses of nerves foreign to a muscle which had formed under experimental conditions became non-functional when the muscle was reinnervated by its native nerve (Marrotte and Mark, 1970a,b; Mark and Marrotte, 1972; Mark et al., 1972; Cass, Sutton and Mark, 1973). The most recent experiment of the series which employed intracellular electrodes showed that this was due to a failure of transmission in the foreign nerve endings although synaptic contact was retained by them (Mark, 1975). The process has been termed synaptic repression by these workers. No information is available concerning the state of trophic interaction at these synapses though if this also were repressed, and the mechanism of repression were the same in developing neuromotor systems, then the effect of such repression on developing moto-neurones would probably be to cause their death. However a similar type of study in which hyperneurotisation of adult rat soleus muscle fibres occurred failed to demonstrate any suppression of the foreign synapses (Frank et al., 1975).

CHAPTER 2

MATERIALS AND METHODS

2.1 Introduction

The technique employed in this thesis makes use of the phenomenon of retrograde axonal transport. Label injected into a region containing nerve endings is transported by the retrograde axonal transport system to the cell bodies with endings in that region. This is therefore a direct method of determining which cells innervate a region.

Although the retrograde movement of vesicles within axons was described as long ago as 1953 (Hughes, 1953) it is only within the last eight years that the important ability of axons of many types to transport various materials from the periphery to their cell bodies has been properly recognized (e.g. Kerkut et al., 1967; Watson, 1968; Kristensson et al., 1971; LaVail and LaVail, 1974; Hendry et al., 1974). Materials are taken up by pinocytosis mainly in the region of the terminal (Zacks and Saito, 1969; LaVail et al., 1973; LaVail and LaVail, 1974) though there are some reports of significant uptake by axon shafts of low molecular weight materials (Feder, 1971; Singer et al., 1972). The rate of uptake by motor nerves can be greatly increased by suitable nerve stimulation (Holtzman and Freeman, 1971; Litchy, 1973). Uptake by free nerve endings has been demonstrated in vitro (Bunge, 1973) but has not been convincingly shown in vivo though suggestive evidence has been obtained in rat hypoglossal nerve (Cull - personal communication).

The microtubule system is probably required for retrograde transport since it is prevented by drugs which interfere with microtubular function (Kristensson and Sjöstrand, 1972; Edstrom and Hansen, 1973; LaVail and LaVail, 1974). It is also prevented by substance interfering with normal oxidative metabolism (Edstrom and Hansen, 1973). The speed of transport has been calculated variously from 15 mm per day in the snail (Kerkut et al., 1967) to 84 mm per day in the chick visual system (LaVail and LaVail, 1974) and 120 mm per day in the rabbit hypoglossal nerve (Kristensson et al., 1971). The intra-axonal route of transport has been proved histologically (LaVail and LaVail, 1974) and experimentally (Kristensson et al., 1971; Kristensson and Sjöstrand, 1972; LaVail and LaVail, 1974).

Horseradish Peroxidase (HRP)(m.w. = approx. 40,000) has been used in the present study. This substance is a stable and active enzyme over a wide range of conditions (British Drug Houses - personal communication), is readily taken up by motor nerves (Kristensson et al., 1971; Kristensson and Olsson, 1971a; Litchy, 1973), is easily detectable in low concentration histologically (Graham and Karnovsky, 1966) and its behaviour in experiments on retrograde transport fairly well documented. When injected as small volumes of concentrated solution (10% w/v) it can be kept well localized (LaVail et al., 1973; LaVail and LaVail, 1974). Nearly all significant uptake takes place at the region of the nerve terminal and not at the axon shaft either in mature nerve trunks in which the perineurium (Watson, 1968; Olssen and Kristensson, 1973; Soderfeldt

and Olsson, 1973) and the myelin sheath (Hirano et al., 1969; Feder, 1971; Krishnan and Singer, 1973) prevent free access of HRP to the axon shaft, or in unmyelinated fibres (LaVail et al., 1973; LaVail and LaVail, 1974).

However HRP can be pinocytosed by myelinated axon shafts although the significance of this in relation to uptake at the endings has not been reported on (Holtzman and Peterson, 1969; Krishnan and Singer, 1973). Whether or not uptake of peroxidase takes place from the shafts of developing fibres is not known. If the axon or any other part of the cell membrane is damaged in the vicinity of HRP then a diffuse stain of the cytoplasm results (Becker et al., 1968; LaVail and LaVail, 1974; Turner and Harris, 1974). This is confirmed in the present study.

HRP remains distinguishable at the injection site for up to four days in the chick optic tectum though it may remain in the extracellular space for considerably less time. HRP was gone from the extracellular space 24 hours following an intracortical injection in the mouse (Turner and Harris, 1974). It may be available for uptake for even less time than this. In a light microscope study of HRP injections into mouse intercostal muscle, no HRP filled vesicles were seen forming as little as one hour after the injection during which time large numbers of HRP filled vesicles had formed (Zachs and Saito, 1969). In the same study, after 24 hours, HRP activity was visible only in the T-tube system of the muscle.

HRP becomes undetectable in chick retinal ganglion cells three to five days after injection into the contra-

lateral tectum (LaVail and LaVail, 1974) and in mouse hypoglossal neurones, by eleven days after injection into the tongue (Kristensson and Olsson, 1973).

2.2 The Experimental Animals

2.2.1 General

Larvae and juvenile of the South African clawed toad, Xenopus laevis were used in all experiments. The advantages of using this animal are:

1. It is easily bred and cared for all year round.
2. Large numbers of offspring are produced in each batch giving large equal-age populations.
3. Larval development is relatively prolonged with good temporal separation of developmental events.
4. The larva is free swimming throughout the development of the limb and limb neuro-motor system allowing easy access at all stages of development.
5. The structure of the spinal cord and the development of the limb is similar to that found in higher vertebrates.
6. A good background of information is available on the development of its spinal cord and limb bud.

The disadvantages are:

1. Its rate of development is sensitive to many environmental factors.
2. Its phylogenetic distance from the human on the vertebrate scale is considerable.

2.2.2 Taxonomic position

The taxonomic classification of Xenopus laevis is as follows:

Class:	Amphibia
Sub-Class:	Apsidospondyli
Order:	Anura
Sub-Order:	Opisthocoela
Family:	Pipidae
Sub-Family:	Xenopodinae
Genus:	Xenopus
Species:	Xenopus laevis

This classification has been taken from Nieuwkoop and Faber, 1956, who also give a detailed historical account of its compilation.

2.2.3 Care of animals

Adult toads were kept in large plastic buckets usually in male and female pairs. The water temperature was maintained between 20° to 23° centigrade and changed every week using fresh tapwater. Their diet consisted of fresh minced liver fed once a week. No other care was required.

Eggs and tadpoles were kept in smaller plastic buckets in aerated tap water at the same temperature. The water was changed two to five times weekly. Larvae were fed from three days after hatching onwards on a diet of beef and liver baby soup strained through a sieve, or spinach powder. Enough food was added to make the water cloudy but not opaque. Food was given after every change of water.

Juveniles as they developed were transferred to tap water in similar plastic buckets. They were fed a diet of tubifex worms once weekly and the water was also changed once weekly.

Special Care Procedures

Tadpoles reared for maximum growth rate were kept at not more than fifty per bucket in water at 23°C. Water changes and feeding were carried out five times weekly.

Tadpoles used for the starvation series were taken from a maximum growth rate batch at Stage 51 and transferred to clean tap water at 23°C. No food was given. The water was changed daily for seven days except the last two days in order to remove faecal material, a possible source of nutrition.

2.2.4 Breeding procedures

Chorionic Gonadotrophin (Pregnyl, Organon) was injected into the ventral abdominal lymph sac by entry from the thigh and through the septum between the thigh and the lymph sac. The doses given were 500 units to the female and 350 units to the male. Injections were given in the evening and the animals placed in a covered bucket for spawning early next morning. Room temperature was maintained at 20 - 23°C. Eggs were removed soon after spawning to aerated water to prevent them being eaten.

2.2.5 Handling of animals during experimental procedures

All utensils coming into contact with animals that were to be kept alive were freshly washed in running tap

water. Operating instruments such as needles, scissors and forceps were flame-scorched. All procedures where animals were to be kept alive were carried out in or using water from the bucket in which they had been kept, and following the procedure, the animals were returned to water of the same source. Fresh tap water was never used for these purposes owing to its apparently deleterious effect.

Animals were paralysed when necessary in the drug MS222 (Sandoz,) at a concentration of about 1:7000 in water for small tadpoles and about 1:5000 for large tadpoles and juveniles. Paralysis was readily reversed within a few minutes of returning to water, which was heavily aerated to keep up constant movement within it.

During procedures where it was necessary to have animals out of water they were placed after paralysis either on a glass microscope slide or glass plate or on a newly poured and cooled paraplast bed moulded to hold the animal in any desired position. With lengthy procedures animals were prevented from becoming dehydrated by having them lying in a small pool of MS222 solution and constantly wetting the exposed skin.

2.2.6 Staging of animals

All animals were staged according to the criteria given by Nieuwkoop and Faber, 1956. Total body lengths were measured using the tip of the tail as the caudal reference point in all animals. A millimeter rule was used. Hind limb lengths were measured using the calibrated graticule of

a dissecting microscope. Calibrations were carried out against a millimeter rule. The proximal reference point of the limb was a point half way along the line of the junction of the limb with the body wall on the lateral surface. Hind limb and forelimb shapes were estimated by direct reference to the figures given in Nieuwkoop and Faber. Forelimbs were not measured. The essential features of each stage are given in Fig. 1.1.

Unless otherwise stated, all staging was done at the time of fixation. and it is to this staging to which data is referred.

2.3 Injection Procedure

2.3.1 Limb injections

Injections were made using hand drawn glass pipettes with shank diameters of 4 mm and tip diameters of 20 to 40 μm. The pipette was filled with a concentrated solution of HRP (10% w/v) by sucking it through the tip. The volume of HRP sucked into the pipette was sufficient to allow slight outflow of HRP when the pipette was held vertically with the tip just below the surface of water thereby preventing capillary uptake of water diluting the HRP. The pipette was connected to a rubber tube and fixed in a vertical position on the arm of a micro-manipulator.

Animals were placed in such a way as to provide easiest access to the required site of injection. If necessary, moulded wax beds and pins to hold back the tail were used. All further manipulations were carried out under a dissecting microscope at x8 to x30 magnification.

The skin over the injection site was punctured with an electrolytically sharpened tungsten needle taking great care

to inflict no damage to the rest of the limb bud. With care, the pipette could be introduced through the hole and the tip placed with great precision in the desired site. HRP was introduced by air pressure exerted through the rubber tubing by a 20 ml syringe. With gentle pressure, a small bolus could be observed forming at the tip of the pipette. Pressure was released instantaneously by removing the syringe from the mouth of the tube at the moment the bolus was judged to be large enough.

2.3.2 Injection volumes

Boluses were measured using the eye-piece graticule to obtain an estimate of the volume injected. Typical boluses in young animals were ovoid to spherical with a long diameter of 100 μm giving an estimated volume of less than 0.001 μl.

It is clear that this technique can give only a very approximate estimate of the volumes of HRP injected, especially for the following reasons:

1. only two axes can be measured
2. the bolus is not always of regular shape
3. the bolus is often not clearly visible.

The last two are most applicable in older animals which have defined tissue planes along which HRP can track, and relative tissue and skin opacity compared to the younger animals. However, the choice of injection volume was governed not by a desire for quantitative uniformity but by the necessity to meet the following two requirements:

1. Confinement of the injection to the intended site.

2. Involvement of a sufficiently large number of axon endings to produce a pattern of distribution of labelled cells in each animal.

The former requirement was most relevant in the youngest animals and this was checked histologically in all animals of Stage 53 or less and all but two of Stage 54 as described below. The latter requirement was most relevant to the older animals and progressively larger injections were given as the limb increased in size in order to accomplish this.

Not only did these considerations rule out the possibility of giving the same volume of injection at all stages, but it was also impossible from a practical point of view to give injection volumes as a fixed ratio of the limb volume, ruling out a quantitative approach from this angle as well. In addition, even if the apparatus had been designed to give exact volumes, use could not have been made of this facility since the same site in different animals accommodates very variable volumes before the HRP threatens to extend beyond the desired site. In addition variable amounts of HRP leak out after the pipette is withdrawn, further adding to the indeterminateness.

2.3.3 Injections in region of spinal nerves

Punctures of the skin were made directly lateral to the notochord opposite the lumbar swelling which is clearly visible in the living Stage 52 animal. At the same time the needle was inserted as far as the notochord three or

four times at slightly different angles in order to damage the perineurium of the spinal nerves. The pipette was inserted to approximately the depth of the notochord and as it was withdrawn, HRP was continually injected. The latter procedure was repeated twice.

2.4 Sciatic Nerve Section in the Juvenile

A small longitudinal incision was made over the neurovascular bundle on the dorsal surface of the thigh, rostrally.

An approach was made to the sciatic nerve through the overlying muscle bellies. Care was required to avoid injury to the large artery and veins running beside the nerve and also to see that the whole nerve was picked up by the forceps. At this point, the sciatic nerve is easily split into its two compartments and one may be missed if not looked for. Section was by crush and cut. The cut ends were reinserted into the wound in rough apposition but no deliberate attempt was made to place them end to end. The wound was not sealed but the natural elasticity of the tissues closed it adequately. The toads were transferred to a shallow tilted bucket of half strength holtfreter solution (see below). The shallow water is required for a few days to allow the animals to learn to swim with one paralysed limb without drowning.

On recovering consciousness, animals were examined for any signs of muscular activity below the knee by carefully looking for movements of the foot to ensure that both bundles of the sciatic nerve had been sectioned.

2.5 Dissection and Fixation

Animals were anaesthetised in greater than 1:1000 MS222 and killed by dissection. Up to Stage 54 the trunk region and limbs were dissected and skinned in one piece and fixed by immersion for four hours in 2% buffered glutaraldehyde plus 5% sucrose at 4°C. (See 2.14.3)

Animals of Stages 54, 55 and 56 were treated as above and in addition excess tissue was removed from around the vertebral column. In animals of Stage 59 and juvenile the spinal cord was dissected out taking care to sever each dorsal and ventral root to prevent the outer layer of white matter being stripped away on removal of the cord from the vertebral canal. During the dissection, the vertebral column was repeatedly immersed in fixative to minimise the delay between death and fixation. The limbs were removed separately for separate treatment. Both limbs and spinal cords were fixed in the same fixative for the same length of time as that used for younger animals.

2.6 Histochemical Treatment of Tissue Blocks*

All tissues for histochemical treatment for locating HRP were washed at least overnight in running tap water at room temperature. Phosphate buffered di-amino-benzidine

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* A few animals early in this work were fixed and treated for HRP demonstration according to a slightly different schedule. These animals were some of those used in Chapter 5 (5.2) and in Lamb (1974) where the schedule then in use is described. The changes incorporated since have resulted in the much more reliable schedule described above.

tetra-hydrochloride (DAB) in a 0.08% w/v solution was mixed and allowed to stand for at least one hour after which the blocks were immersed in it. After a period ranging from 1 to 4 hours depending on the size of the block, hydrogen peroxide was added to make a 0.01% w/v solution and the blocks left to stand for a further period of time equal to the first. Solutions were at room temperature and occasionally agitated. The blocks were then either transferred to alcohols for embedding immediately (see below) or to water for storage in the refrigerator at 4°C before embedding the following day.

2.7 Decalcification Procedure

Stage 59 and juvenile limbs were decalcified in ethylene-diamine-tetra-acetic acid (EDTA) at 4°C for one week after histochemical staining. Following decalcification they were embedded in wax as described below.

2.8 Embedding Procedures

2.8.1 All blocks except spinal cords

Schedule

70% cellosolve	¾ hour
100% cellosolve	½ hour
100% cellosolve	1 hour
100% amyl acetate	¾ hour
paraplast (M.P. 55°C) at 65°C	3 hours

rapid solidification in mould in cold water.

2.8.2 Spinal cords

Schedule

70% cellosolve	½ hour
100% cellosolve	1 hour
100% amyl acetate	½ hour
paraplast (as above)	2 hours

rapid solidification in mould in cold water.

2.9 Preparation of Slides for Microscopy

Blocks were cut into ribbonned serial transverse sections 10 μm thick on a Reichart microtome. These were arranged on the slides with strict attention to serial order with as many sections per slide as possible, to facilitate mapping (up to 300 sections per slide). The majority of tadpoles and all spinal cords were thus prepared on one slide each. After being allowed to dry for at least 24 hours, slides were dewaxed in xylene, mounted in DPX and stored at room temperature for about two weeks to give optimum conditions for detecting the label by phase microscopy.

2.10 Mapping of Labelled Cell Distributions

2.10.1 Microscopy and form of map

All mapping was done under phase microscopy at X400 with white light. After two or more weeks storage of the slides most histological detail within the spinal cord becomes faint in these viewing conditions. Peroxidase granules stand out very clearly as dark dots, looking quite black at their most intense. Diffusely stained cells

show as grey or black though the most intensely stained cells also show the brown colour characteristically seen with normal optics. With normal optics, all peroxidase labelling is still visible though often faint and difficult to see when quickly scanning the necessary hundreds of sections.

For mapping purposes only cells with grainy label were counted except where otherwise stated. Labelled cells were plotted on graph paper ruled into three columns representing the lateral, middle and medial parts of the ventral horn as seen in transverse section. Vertically, each graph paper division represented one tissue section with the most rostral sections represented at the top of the page and caudal sections at the bottom (Fig. 2.2).

2.10.2 Identification of the rostral end of the ventral horn

The rostral end of the ventral horn was identified using the following criteria:

1. In animals of Stage 55 or more, by the direct identification of ventral horn cells. Because of the slight danger of confusing the most rostral of these with primary motor neurones, the ventral horn was identified at more caudal levels and traced to its rostral end through serial sections.

In younger animals:

2. The most rostral identification of circumferentially oriented cells.

3. The appearance of cells lying outside the smooth convex profile of the grey matter seen rostral to the ventral horn.

4. The rostral end lies rostral to the most rostral limb sensory ganglion (usually ganglion 8) but rarely as far rostral as the next ganglion containing large ganglion cells characteristic of thoracic ganglia.

5. The presence of intracellular peroxidase label.

As for older animals, the identification of the rostral end was aided by tracing the ventral horn through serial sections from a more caudal level. In some animals in which a single point in the ventral horn could be identified with accuracy to within one section, repeated identifications of the rostral end agreed in nearly all cases to within less than five sections (or 2 to 5%).

Having established the position of the rostral end, sections were counted moving caudally. Labelled material was scored differently in animals up to Stage 54 from those of Stage 55 or more.

2.10.3 Method of scoring in older animals

Over Stage 54, cells with label in the perikarya were counted with no allowances made for double counting though cells were only counted if the nucleus was visible. In animals of Stage 59 and juvenile only cells containing a nucleolus were counted. Four cells or more in one medio-lateral subdivision of the ventral horn were counted as four since only in Stages 55 and 56 could more than four fit into one part of one section, and in these cases the smaller cell size led to overlapping and difficulty in distinguishing individual cells in fours or more.

2.10.4 Method of scoring in younger animals

At Stage 54 and below individual cells could not be accurately counted because of overlapping and the difficulty of ascertaining the precise location of label, whether in the thin perikaryon or the dendrites. In these cases label was graded from one to four according to its profuseness. Grade one was equivalent to individual labelled cells which could be seen and Grade 4 equivalent to the most profuse labelling seen in any animal, although this probably involved more than four labelled cells. Thus the scale was absolute and not adjusted to each animal. These grades therefore gave an indication of the actual labelled cell number though there was undoubtedly considerable underestimation in densely labelled areas becoming less important with increasing stage and cell size. Conversely, overestimation due to the counting of intra-dendritic label would have occurred in all areas but also become less important with increasing stage.

2.10.5 Determination of medio-lateral distribution

Cells or label were only assigned to medial or lateral compartments if they were clearly so, thus the middle compartment was larger than either medial or lateral, the approximate ratios of medial:middle:lateral being 1:2:1. As a result of this, a medial or lateral tendency of a pattern had to be quite definite before it would be recorded.

2.10.6 Plotting of scores

Using these criteria for scoring, a score was inserted

in each medial, middle and lateral compartment of each vertical division of the graph paper in order down the page corresponding to the rostro caudal order of serial sections. In this way a map of distribution and density of labelled cells was constructed for every ventral horn under study and the term map or maps refers to these.

2.10.7 Identification of caudal end of ventral horn

The caudal end of the ventral horn was identified by the same criteria as used for identifying the rostral end except that criterion four was suitably altered as follows.

The caudal end of the ventral horn usually lies caudal to the most caudal limb sensory ganglion which is usually ganglion 10 but rostral to ganglion eleven which is characterised by its small size and large cells.

The error of identification was found to be no greater than for the rostral end using the same method of determination of error.

2.11 Identification of Injection Site

In all animals except Stage 58 and juvenile, transverse serial sections of the limb were included on the same slide as that containing the ventral horn. Limbs of the older animals were prepared for microscopy as described in 2.8. These were also cut in transverse section. Limb sections were examined with normal optics using white light since cellular detail remains prominent by phase microscopy even after storage, and label is not visible, in contrast to the situation in the spinal cord. Injection sites were examined

Fig. 2.1

Criteria for assessing localization of injection site.

Very well localized:	Injection site small, circumscribed, well within specified region.
Fairly well localized:	Injection site large, or near edge of specified region, or both.
Fair:	Injection site mainly within specified area. Small amount encroached on another region.
Poorly localized:	Injection site involving large areas beyond specified region.

in all sections passing through them to ensure that no spread of the HRP to regions other than that specified had occurred. No attempt was made to map the precise boundaries of the injection site and only a qualitative grade of localization to the correct site was given ranging from very well localized to poorly localized (Fig. 2.1). These assessments were all written on the maps themselves.

2.12 Derivation of Grids

2.12.1 Selection of maps

Only the maps of those animals which had an injection site classified as very well localized or fairly well localized were used. Animals with injection sites classified as fair or poorly localized were not included.

2.12.2 Types of grids

Two types of grids were derived:

1. Averaged percentage distribution grids.

These were designed to show the average projection pattern of the animals of a group

2. Distribution variation grids.

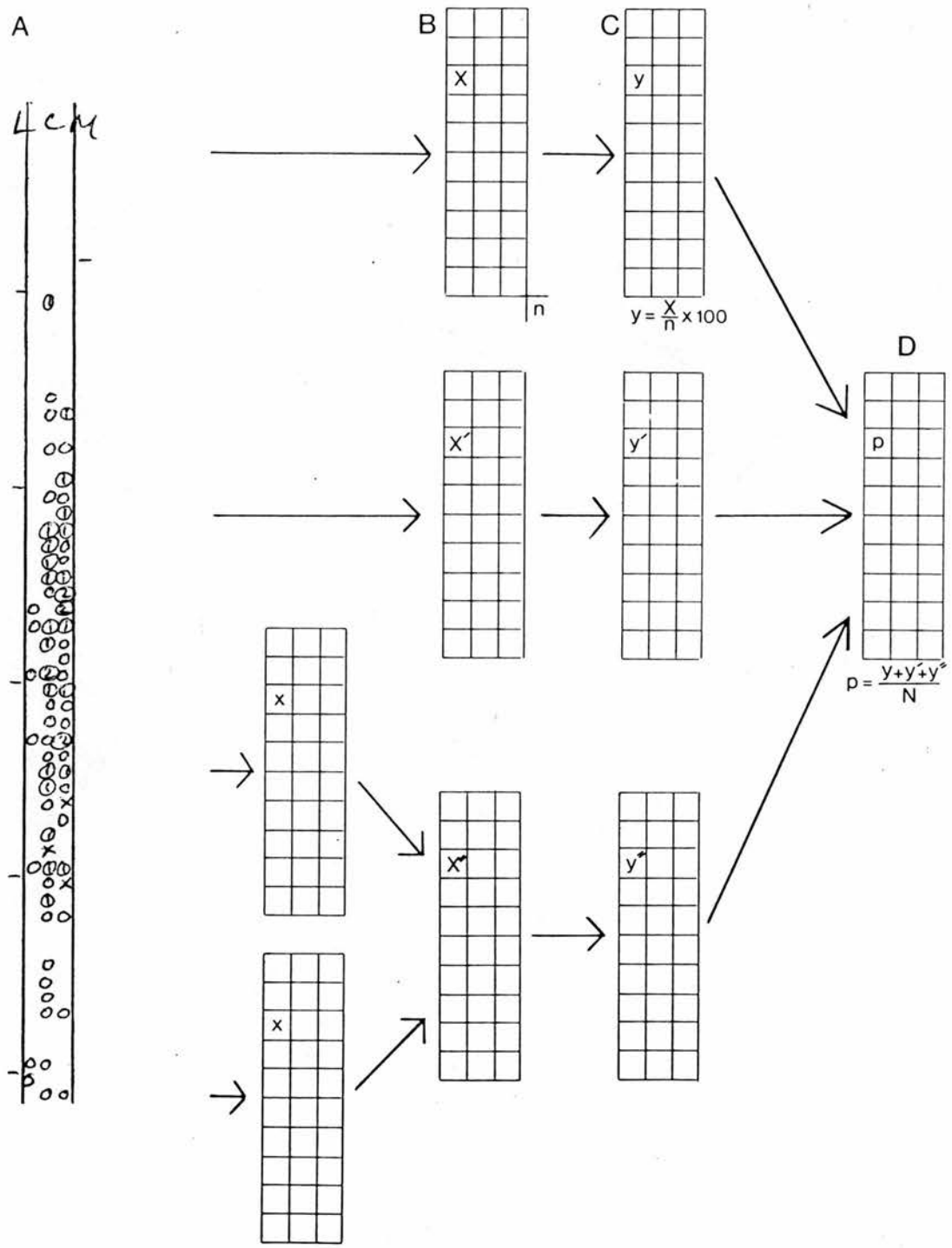
These were designed to show the degree of pattern variation between individual animals of a group.

2.12.3 Derivation method (Fig. 2.2)

Maps were divided into ten equal segments rostro-caudally and medial, middle and lateral labelled cells

Fig. 2.2

Method of deriving averaged percentage distribution grids. A: Example of part of a map (Juvenile PMT). B: individual cell distribution grid (one per map). C: individual percentage distribution grid. D: averaged percentage distribution grid. LCM: lateral, central and medial. X: number of labelled cells in one tenth of one column of the map. n: total number of labelled cells in map. N: number of individual percentage distribution grids making up the averaged percentage distribution grid . Individual cell distribution grids with $n < 10$ (bottom left pair of grids) are combined ($X'' = x + x$) before deriving percentage values.


A
4 cm
B
X
n
C
y
$y = \frac{X}{n} \times 100$
D
p
$p = \frac{y+y'+y''}{N}$
X'
y'
x
x
X''
y''

counted in each tenth. Totals were placed in correct order in a 3 x 10 array corresponding to the three medio-lateral subdivisions by the ten rostro-caudal subdivisions. These arrays are the individual cell distribution grids. These were then converted to individual percentage distribution grids by calculating each cell number in the grid as a percentage of the total cell number within the grid. In turn, these were averaged to give the averaged percentage distribution grids. The purpose of the conversion to percentages was to remove the effect of varying labelled cell numbers in different animals. However, with low cell numbers, individual cells took on a disproportionate importance. For this reason maps with less than ten labelled cells were added together before conversion to a common percentage distribution grid which was then treated in the same way as the other individual percentage distribution grids.

To obtain the distribution variation grid (Fig. 2.3) each individual percentage distribution grid in a group was assigned a different letter. Every percentage number in an individual percentage distribution grid is represented by that grid's letter in the corresponding box of the distribution variation grid. The letter may be upper case to represent major percentages or lower case for minor percentages. Minor percentages totalled less than 10%. Each box in the distribution variation grid has room for the letters of up to nine individual percentage distribution grids. If there are more than this in a group, the nine most extreme variants are included.

Fig. 2.3

Method of deriving distribution variation grids using a group of three juvenile PMT results as an example. Each percentage value of the individual percentage distribution grid is represented in the corresponding box of the distribution variation grid by the letter assigned to that individual. Major percentages are in upper case, minor in lower case.

Individual Percentage Distribution Grids of Three Juvenile PMT Maps

(A)

	4	2
	7	7
	13	12
1	13	11
	9	6
	5	3
1	1	2
	1	

(B)

	4	4
3	15	18
2	17	16
1	8	5
1	4	2

(C)

		12
	8	16
	24	4
4	20	4
		4
	4	

Calculation of major (upper case) and minor (lower case) percentages in individual percentage distribution grid A:

MINOR		Total
4	Values of 1%=4	4
2	Values of 2%=4	8
Cut-off point		
1	Value of 3% =3	11
MAJOR		

Distribution Variation Grid for the three juvenile PMT maps

	A	a
	AB	AB
b	AB	ABC
ab	ABC	ABC
b	ABC	ABC
bC	ABC	AbC
a	a	a C
	a C	

2.13 Quantitative Values in this Work

The total numbers of labelled cells in each map were used to derive an estimated mean labelled cell number and standard error for every group.

Ideally, the mean and standard error should accurately reflect the number of ventral horn cells innervating the injection sites within the group. However this number is affected by various factors.

1. Not all ventral horn cells necessarily transport HRP.
2. Not all ventral horn cells that do transport HRP will necessarily be detectable if the quantity transported is below a threshold which may vary from animal to animal depending on the penetration of histochemical reactants (see 3.2.2).
3. Counting of labelled cells is affected by a tendency towards overestimation in all areas and a tendency towards underestimation in densely labelled areas (see 2.9.4). Apart from leading to an unquantifiable error in labelled cell numbers, the latter error also leads to a flattening of the estimated cell distribution curve, and the former to an exaggeration.

Each of these factors varies in relation to the stage of the animal so that the relationship of the estimated mean to the true mean (i.e. mean number of cells actually innervating injection site) varies in an unquantifiable manner with different stages, making comparison between estimated means of groups of different stages hazardous.

The number of labelled cells in a map may not only be varied by the above factors which refer only to the number

of cells innervating the injection site, but also by variations of the size of the injection site itself. That is, the true means may vary for non-physiological reasons. As mentioned earlier, this varies both absolutely and relative to limb size with successive stages as well as varying within individuals of a group (2.3.2). Although the latter type of variation is catered for in estimating the means and standard errors, the former type of variation also makes hazardous the comparison of estimated means of groups of different stages.

The result is that no information can be obtained about changes of innervation density of a given region with age, since the changes of cell number which might result from this would be obscured by all the other ways in which labelled cell number can change with development. However if the reasonable assumptions are made that all the errors discussed affect the cell numbers for each region to a similar degree at equivalent stages, and that the injection site sizes in the two thigh regions or the two distal regions at equivalent stage are about equal then comparison between the means of the two thigh regions or the two distal regions, or two different groups of the same region at equivalent stage are more valid. Such comparisons are made when appropriate using students 't' test.

2.14 Solutions and Fixative

2.14.1 1/15 molar phosphate buffer pH7*

Mix

Sodium dihydrogenphosphate ($NaH_2PO_4 2H_2O$)	12.48 gm
Disodium hydrogenphosphate ($Na_2HPO_4 12H_2O$)	42.96 gm
Distilled water	3 ℓ

1/30 molar phosphate buffer of pH 7 was made by combining equal volumes of 1/15 molar buffer and distilled water.

2.14.2 Buffered 5% sucrose solution

Mix

Sucrose	50 gm
1/15 M phosphate buffer	1.0 ℓ

2.14.3 2% glutaraldehyde fixative

Mix

Glutaraldehyde 25%	4 ml
Buffered 5% Sucrose	50 ml

2.14.4 10% Horseradish peroxidase solution

Horseradish peroxidase powder (British Drug Houses)	30 mg
1/15 M phosphate buffer	0.3 ml

Float horseradish peroxidase on top of buffer in a watchglass. Allow to dissolve without stirring but only tipping watchglass. After dissolving store in refrigerator ($4^{\circ}C$) at least overnight before use to disperse any lumps.

* pH7=hydrogen ion concentration of $_{100}$ n mole/litre

Solution retains full activity for at least five weeks if stored in refrigerator.

2.14.5 Diaminobenzidine (DAB) and hydrogen peroxide solution

Mix

DAB (tetrahydrochloride salt - British Drug Houses) 50 mg

1/30 M phosphate buffer 60 ml

When required (see 2.6) add

Hydrogen peroxide (20 vols. per vol.) 0.03 ml per 10 ml of solution

(gives 0.01% H_2O_2)

2.14.6 Holtfreter solution

Five times normal concentration Holtfreter solution was prepared as follows:

Mix

Sodium chloride	17.5 gm
Potassium chloride	0.25 gm
Calcium chloride	0.5 gm
Sodium bicarbonate	1.0 gm
Distilled water	1 litre

Half strength Holtfreter was prepared by mixing 1 volume of concentrated solution with nine volumes of water from the bucket containing the juveniles.

2.14.7 Ethylene diamine tetra acetic acid (EDTA) solution

Mix

EDTA (sequestrene, di-sodium salt) 250 gm

Distilled water 1750 ml

Adjust to pH 7 by addition of sodium hydroxide pellets.

CHAPTER 3

RESULTS OF PRELIMINARY OBSERVATIONS AND EXPERIMENTS WITH HORSERADISH PEROXIDASE (HRP) UPTAKE

3.1 Introduction

These observations and experiments were required to clarify the behaviour of HRP, in the limb and spinal cord, and the significance of the various forms of brown stain seen in the spinal cord after the histochemical treatment with DAB and hydrogen peroxide. In addition it was necessary to understand the modes of uptake of HRP that could occur in order to ascertain the usefulness of HRP in conducting the types of experiments subsequently carried out. Much of this work merely seeks the necessary confirmation that principles found in other systems and other species as described in the introduction to Chapter 2 are also applicable to the neuromotor system of the Xenopus limb.

3.2 General Description of Effects of Injecting HRP into limb 44 hours before fixing

3.2.1 Limb

The site of the injection is indicated in two ways:

1. The localized presence of red-blood cells and peroxidase containing macrophages. Both show a deep brown stain (Fig. 3.1 A-D).

2. Fine granules of brown material about 1 μm diameter dispersed throughout the tissue in the area of the injection (Fig. 3.1 A-D).

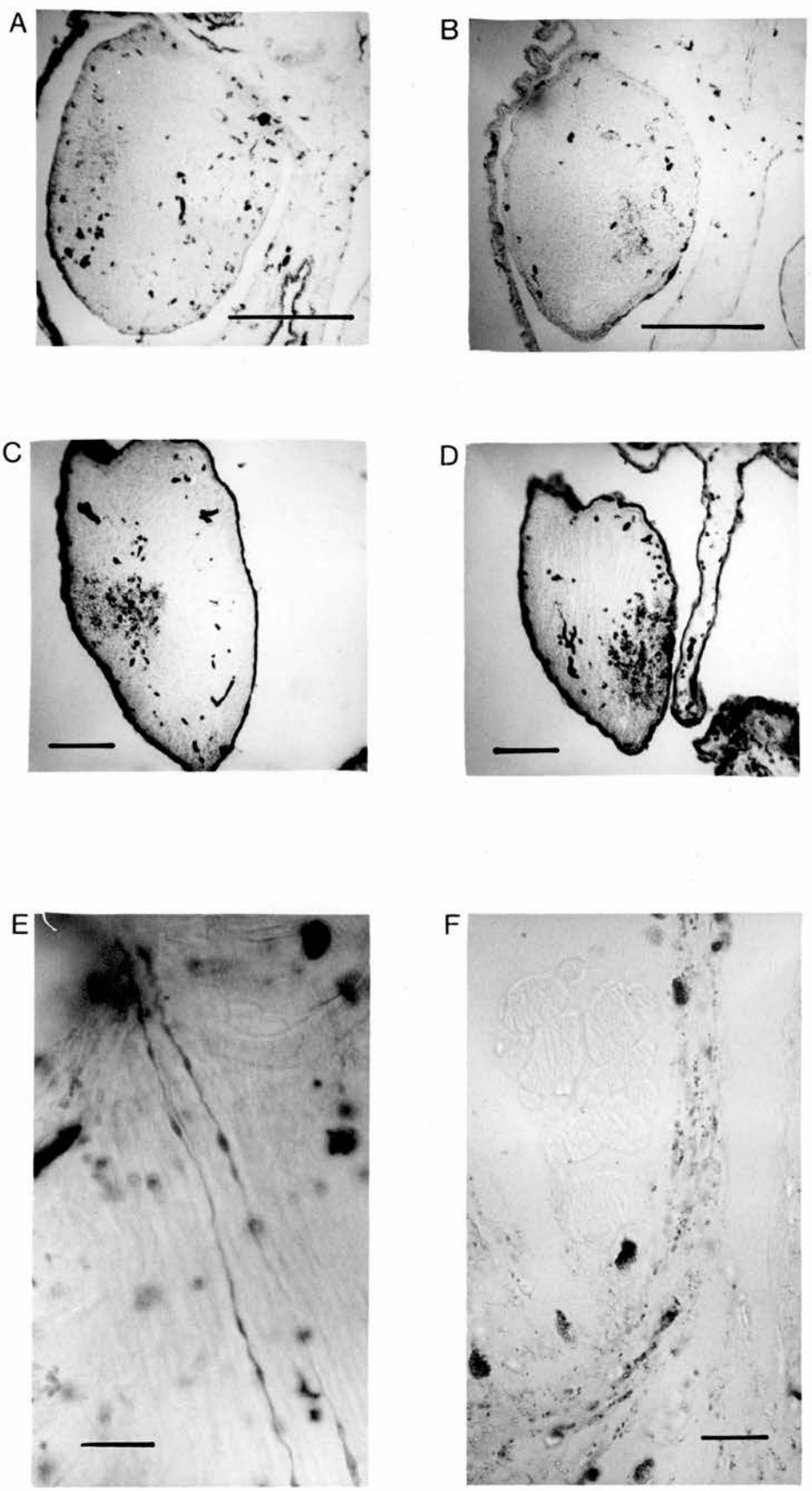
Depending on the size of the injection and the degree

Fig. 3.1

A-D: Sites of horseradish peroxidase injections after 44 hours. Site is visible as a collection of very fine granular material. Stained red blood cells and macrophages may also be collected in clumps at the site of injection (collections of large black dots in C and D). A: ALT, Stage 50-51. Scale = 200 μm. B: PMT, Stage 50. Scale = 200 μm. C: ALA, Stage 53. Scale = 100 μm. D: Plantar, Stage 52. Scale = 100 μm.

E: Diffusely stained axons in the IXth spinal nerve in the region of the notochord of a Stage 54 animal after HRP injection into limb 44 hours before killing. These fibres were traceable from the limb to the ventral horn in serial 50 μm sections. Swellings may represent early degenerative changes. Scale = 10 μm.

F: Mottled and coarse granular material in the IXth spinal nerve where it leaves the base of the limb (lower left) after HRP injection into limb 44 hours before killing. Stage 51. Scale =30μm.


A
B
C
D
E
F

of trauma, each of these can vary considerably in localization and intensity. Well localized injection sites contain few red blood cells or macrophages and the fine material is clearly confined to a small volume of down to approximately 100 μm in diameter (Fig. 3.1 A-D). Less well localized injections may show a greater or lesser spread of each of these forms of staining relatively independently, either diffusely over a larger area or following well localized tracts through the tissues. In older animals, these tracts tend to follow tissue planes.

Further brown stain is also visible as:

1. Scattered red blood cells generally within capillaries throughout the limb.

2. Diffuse non-specific brown stain affecting all tissues with equal intensity extending in from the surface of the limb for a variable distance, the ectoderm and immediately subjacent mesoderm being most affected. The degree of this stain depends on the time of incubation of the specimen in DAB. In heavily overstained limbs, the site of the injection is often not distinguishable.

Both of these latter types of stain occur throughout the block and in animals which have received no injection of HRP or an injection of water.

3. Mottled and granular material in nerve trunks leaving the area of the injection. This tends to lie in coarse strings running parallel with and intermingled within the trunk (Fig. 3.1 F). This appearance is never seen in uninjected limbs or in animals receiving a water injection.

These observations show that it is possible to define the extent of the spread of HRP in the injection site so that animals with injections not satisfactorily confined to the regions specified in later experiments can be rejected from further analysis.

3.2.2 Spinal cord

In all animals, experimental or control, when the specimen has been incubated for the correct amount of time, all tissues of the cord apart from the localized forms of staining to be described below show an almost white appearance with a very faint brown discoloration. Over-incubation results in a diffuse brown stain affecting all tissues equally, such diffuse stain tending to obscure the localized forms.

The localized forms of staining occur as follows:

Control animals

In those animals receiving no injection or a water injection and processed in the usual way for peroxidase activity:

1. Red blood cells, scattered randomly within capillaries throughout the cord (Fig. 3.3 A). In specimens which have been incubated for insufficient time, red blood cells fail to stain, the centre of the cord being worst affected. These specimens were always discarded from further analysis.

2. Coarse intra-cytoplasmic granules up to about 2 μm in diameter in some primary motor neurones on either side

Fig. 3.2

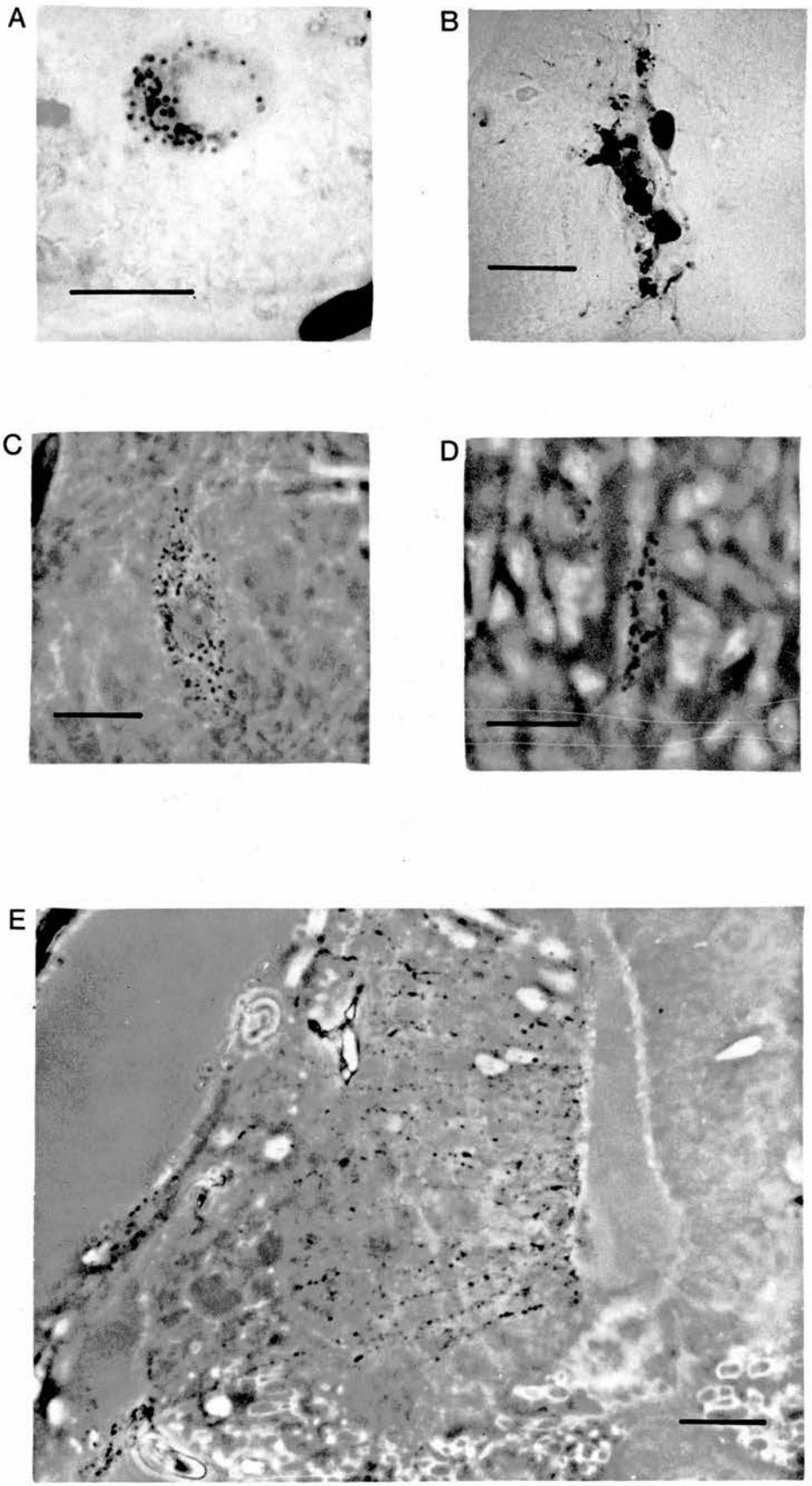
A: Stage 51. Primary motor neurone containing large numbers of brown granules presumed to result from endogenous peroxidase activity in the form of peroxisomes. Sections prepared as on p. 37 et seq. Scale = 40μm.

B: Stage 53. Diffuse cells both healthy and degenerating with end products of previously degenerated cells after HRP injection into limb 44 hours before killing. Sections prepared as above. Scale = 25μm.

C: Grainy cell Stage 59 (Phase contrast)
Procedures as in B. Scale = 30μm.

D: Grainy cell Stage 51 (Phase contrast)
Procedures as in B. Scale =10μm.

E: Stage 51. Transverse section to show radial strings on the side of the spinal cord ipsilateral to a limb injection. They appear to be continuous with the ventral root at the lower left and are also visible in the dorsal root above. (Phase contrast). Procedures as in B. Scale = 30μm.


A
B
C
D
E

of the animal (Fig. 3.2 A). These are assumed to be peroxisomes, a source of endogenous peroxidase activity (Citkowitz and Holtzman, 1973).

Experimental animals

The following types of stain may or may not be present 44 hours after injection of HRP depending on the stage of the animal, the site of the injection and its success. These forms are seen only on the side of the injection.

1. A variable number of diffusely stained ventral horn cells (diffuse cells)(Figs. 3.2 B, 3.3 C, 4.1, 4.2). The intensity of the stain is variable and usually the nucleus is more darkly stained than the cytoplasm. The stain is of even intensity and can easily be seen in dendrites and axons. It is possible to follow individual axons stained in this way through serial 50 μm sections from the spinal cord to the limb (Fig. 3.1 E). Dendrites can similarly be followed to the finest branches that can be resolved by light microscopy and are present even on the earliest labelling cells. A more detailed description of diffusely labelled ventral horn cells at successive stages will be presented later (Chapter 4).

From around Stage 53, a large number of diffuse cells appear to be undergoing degeneration (Fig. 3.2 B).

2. Material composed of grains up to 1 μm in diameter distributed in a well defined pattern. Either it is clearly visible in the cytoplasm of the perikarya and dendrites of individual ventral horn cells (grainy

Fig. 3.3

A: Typical appearance of grainy material in transverse section of the ipsilateral ventral horn 44 hours after a limb injection at stages up to Stage 54. Sections prepared as described on p. 37 et seq. Note typical appearance of red blood cells (top centre, bottom right)

Scale = 20 μm.

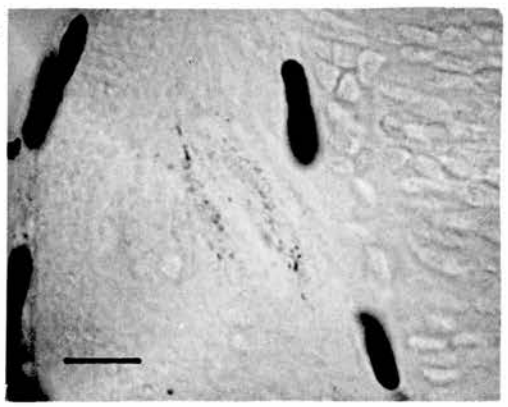
B: Oil immersion view of ventral horn cells with both a light diffuse stain and grainy stain to show manner in which grains are distributed within cytoplasm, after same procedures as in A. Also see fig. 3.2

Left: Stage 52, Scale = 15 μm.

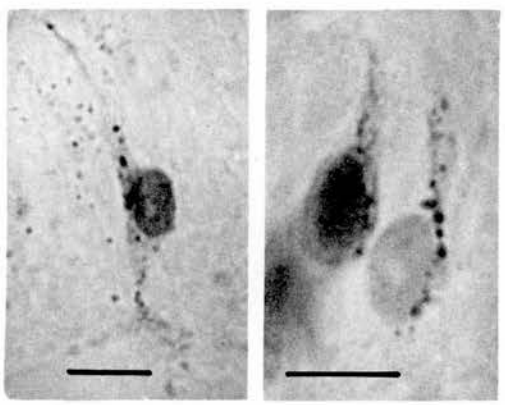
Right: Stage 51, Scale = 10 μm.

C: Labelled cells resulting from bathing the newly sectioned proximal end of the sciatic nerve in horseradish peroxidase in the juvenile 44 hours before killing, and preparing sections as above. Both diffuse and grainy cells are visible. The diffuse cells look unhealthy with eccentric nuclei, varicose dendrites and irregular cell membranes. (Phase contrast). Scale = 40 μm.

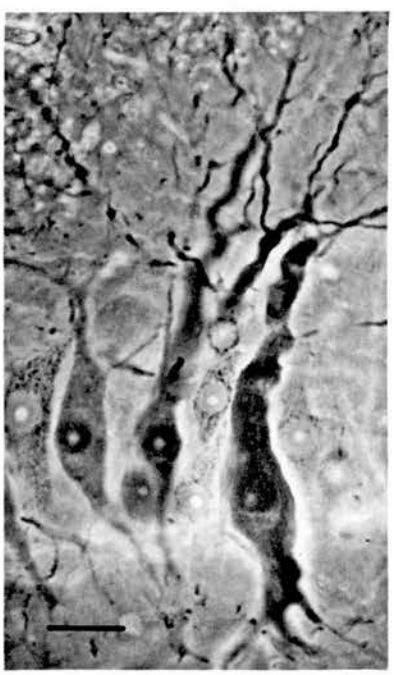
A



B



C



cells)(Fig. 3.2 C,D) or it falls into a pattern consistent with the material being in that location. That is, it is arranged in roughly parallel strings running circumferentially within the ventral horn closely resembling the outlines of diffuse cells and their dendrites (Fig. 3.3 A). The former description applies mainly to older animals with large ventral horn cells containing much cytoplasm whereas the latter description applies to young animals with small ventral horn cells which have only a very thin rim of cytoplasm and which considerably overlap in the section. Very close examination with the highest power lens (x 1000 oil immersion) of the grainy material in the young animals corresponding to the latter description reveals that much of it is lying in continuous strings, clearly intra-cellular which extend from one polar dendrite, around the rim of cytoplasm about the nucleus, often on only one side, to the other polar dendrite (Fig. 3.2 D, 3.3 B). The rest of the grainy material cannot definitely be seen to be intra-cellular although its appearance exactly resembles the intra-cellular material and it is felt safe to assume that it is intra-cellular.

3. A smaller number of cells showing both diffuse and granular staining as described in (1) and (2) above (diffuse and grainy cells). This appearance can be seen at all stages of development but is more prominent in older animals (Fig. 3.3 B).

4. Sometimes, in the ventral roots, grainy material lying in strings running parallel to the nerve fibres

similar to the appearance described in the nerve trunks of the limb (Fig. 3.2 E).

5. In some young animals up to about Stage 53 usually if they have been growing slowly, a large number of strings of granules up to 1 μm in diameter extending radially from the central canal to the pia of the whole experimental side of the cord except the extreme dorsal part (radial strings). Some of the radial strings appear to be continuous with granular material within the ventral root. A small amount of granular material is also often visible within the central canal wherever the radial strings appear (Fig. 3.2 E). The precise anatomical location of the strings is not known.

6. The spinal ganglia on the injected side also contained diffuse and grainy cells but no study was made of these.

All forms of staining described for experimental animals in both limb and cord are visible 18 hours after injection. The diffuse and granular stain within ventral horn cells, and the radial strings are however considerably less prominent.

These observations show that the histochemical technique is satisfactory for demonstrating the presence of peroxidase activity in the spinal cord including the ventral horn cells. Peroxidase activity may result from the presence of HRP or from endogenous peroxidase activity such as is found in red blood cells and the primary motor neurones. The finding that for any individual group of labelled ventral horn cells a precise, specified region of

limb must be involved in an injection of HRP (Chapters 6 and 7) is very strong indirect evidence for a retrograde axonal uptake of HRP into the cell cytoplasm. Further support is given by considering the theoretical possible alternative mechanisms which could produce the peroxidase activity, all but one of which can be dismissed immediately.

1. Haematogenous carriage of HRP to the ventral horn cells - left and right sides should be equally affected.

2. Lymphatic carriage - though it is conceivable that one side may be more affected than the other, this mode of carriage cannot explain how different groups of ventral horn cells are labelled after injection into closely sited limb regions (Chapters 6,7).

3. Transport through the extra-cellular space. Only one part of the extra-cellular space is continuous with that around the ventral horn cells and that is the part around the axons themselves. However, there is nothing to prevent free access of the HRP to the extra-cellular space around all axons equally where they intermingle at the base of the limb. There are no septa or other partitions, and axons freely interchange between bundles (Prestige and Wilson - personal communication). Such a mechanism cannot account for small groups of labelled cells.

4. Induction of endogenous peroxidase activity by the process of injection. This has been ruled out in the water injection controls (3.2.2).

5. Alteration of ventral horn cells by the process of

injection enabling them to take up HRP carried by an extra-axonal route resulting in labelling only of cells with axons affected by the injection. Although this mechanism would be equally useful for projection studies it has been ruled out experimentally (3.3).

6. Transport via sensory fibres and the proximal processes of dorsal root ganglion cells to the ventral horn cells. There is no proof that this does not happen. However this is assumed to be unlikely for the following reasons. Normal uptake of HRP by all nerve fibres examined in all species is within membrane bound structures (e.g. Becker et al., 1968; Kristensson and Olsson, 1971b; Bunge, 1973; Turner and Harris, 1974; LaVail and LaVail, 1974). The grainy label seen in the ventral horn cells in the present study is assumed also to be within similar structures since the light microscopic appearance is the same as that seen in the cell bodies in other studies. In those studies the grains were shown by electron microscopy to be membrane bound structures (Kristensson et al., 1971; Kristensson and Olsson, 1971b; LaVail and LaVail, 1974). Transfer of HRP from a dorsal root ganglion process to a ventral horn cell therefore requires at least the exocytosis of HRP from a vesicle within the dorsal root ganglion process and the pinocytosis of the released HRP by the ventral horn cell. These events would have to occur opposite each other and simultaneously to prevent too great an attentuation of the HRP concentration. It seems unlikely that this could occur

sufficiently often to account for the very profuse labelling of ventral horn cells usually seen. In addition, some HRP would be expected to escape to the extra-cellular space and areas outside the ventral horn. No evidence of this was seen.

A further reason for considering the sensory fibre route unlikely is that frequently, single isolated labelled ventral horn cells are seen which may be separated by hundreds of micrometers from the nearest labelled neighbour. It seems unlikely that the proximal process of a dorsal root ganglion cell should interact in this way with only a single ventral horn cell, whereas it is quite reasonable that an isolated cell should send an axon to the injection site.

The two fundamentally different types of HRP labelling described in ventral horn cells was considered to be due either to different modes of uptake or different modes of intra-cellular treatment of the HRP. This question and the significance of each type of label to projection studies is examined in 3.4, 3.5, 3.6 and 3.7.

The significance of the radial strings is not known. Among the possibilities are:

1. Granules within radial dendrites of labelled ventral horn cells or remnants of the processes attaching them to the central canal. This is thought to be improbable partly because of the appearance of the strings which are very evenly distributed in a large sector of the experimental side of the spinal cord as seen in transverse

section and because the rostro-caudal distribution of the radial strings does not match the rostro-caudal distribution of labelled cells and also because diffuse cells do not show similar dendrites or processes. However, if the dendrites or processes are very small and below microscopic resolution then diffuse radial lines would not be visible.

2. Another type of cell with processes into the limb. None are known of.

3. Inter-cellular transfer of HRP from ventral horn cells to another cell type, for example, glia. Even if this occurs it is unlikely to explain the radial strings for the reasons of the different distributions of radial strings and labelled ventral horn cells given earlier.

4. Extracellular HRP tracking up nerve trunks and lying in the extra-cellular space of the spinal cord. This is supported by the observation that radial strings are usually most pronounced opposite the points of entry of dorsal and ventral roots. This is considered the most likely explanation.

No further study was made of radial strings.

3.3 Experimental Test of the Possibility that Ventral Horn Cells may be Affected by an Injection Allowing them to take up Circulating HRP at the Cell Body

Five animals of Stages 51 to 53 were given injections by the usual method into the left limb bud. At the same time a large hole was made in the right limb bud with the tungsten needle to simulate the physical damage caused by

the injection procedure. The animals were processed and examined in the usual way after 44 hours.

The left ventral horn of all the animals contained the usual complement of diffuse and grainy cells. In four animals the right ventral horn contained no labelled cells. In the fifth, one faint diffuse cell was found.

It is concluded that ventral horn cells do not label by differential uptake of circulating HRP. The single labelled cell may have somehow grown an axon to the contralateral side or, as is more likely, it may have resulted from the introduction into the right limb bud of a minute quantity of HRP which had leaked from the left sided injection and been carried in the drop of water bathing the tadpole during the subsequent needle damage to the right limb bud.

3.4 Effect of Injecting HRP around Freshly Cut Stump of Sciatic Nerve in Thigh of Juvenile

The object of this experiment was to determine whether diffuse cells resulted from damage to the axon.

Five animals were examined, all showing a similar result. After 44 hours both grainy and diffuse cells were present (Fig. 3.3 C), the diffusely stained being present in very large numbers. The majority of cells in the caudal half of the ventral horn and all the cells in the caudal end were diffusely stained. Some were grainy stained and a few unstained. In the caudal part of the rostral half of the ventral horn the proportion of diffuse cells fell with rostral progression and larger numbers of

grainy cells were seen. Further rostrally only grainy or unstained cells were seen and at the most rostral end only a small proportion of cells were stained. Many diffuse cells had an unhealthy appearance with broken varicose dendrites, irregular cell membranes and sometimes eccentric nuclei. These may have represented shrinkage artifacts though grainy labelled cells showed none of these changes.

The axons passing through the point at which the sciatic nerve was broken are mainly destined for regions below the knee since the main branches to the thigh muscles have already separated more proximally (Ecker, 1889). In general, the rostral end of the ventral horn innervates proximally while the caudal half innervates some thigh musculature and the below knee regions. It is likely that the most caudal end of the ventral horn innervates only the distal parts of the limb (Chapter 6). The hypothesis that damage to the axon results in a diffuse stain therefore predicts that in this experiment, large numbers of caudal ventral horn cells and few rostral ventral horn cells should be diffusely stained and that the most caudal ventral horn cells should all be diffusely stained. In addition, since the HRP must have come into contact with thigh musculature in order to involve the broken sciatic nerve, grainy cells should be found in the ventral horn conforming to the distribution of thigh representation. All these predictions are met in this experiment thus supporting the hypothesis.

That the diffuse stain may be the result of regenerating endings growing out from the cut stump is disproved in 3.6. Since there are no other reasonable possibilities, it may be accepted that damage to the axon results in a diffuse stain. However it cannot be concluded that all diffuse cells are necessarily due to axonal damage. (see 3.8)

3.5. The Effect of Injecting HRP into the Left Plantaris Muscle of Juveniles and Sectioning the Left Sciatic Nerve in the Thigh the Following Day

The object of this experiment was to test whether damage to the axon causes the diffuse stain as a result of an altered mode of uptake peripherally or an altered mode of handling of the label within the cell body.

Five animals were examined 24 hours after sectioning the nerve. All animals had moderate numbers of grainy cells in the left ventral horn which appeared to conform to the typical distribution for plantaris motoneurones (Fig. 8.4). However these cells were not mapped. One animal had two diffuse cells and one animal had one diffuse and grainy cell.

During the period between injection and sectioning the nerve, the axons will have been transporting material in the normal manner which results in grainy cells. After sectioning the nerve in the thigh, no further transport of HRP could have taken place. Since the grainy appearance of the label was not changed after 24 hours, it may be concluded that the diffuse stain results from altered uptake at the site of damage and not from an altered mode of handling of the HRP by axotomised ventral horn cells.

The two diffuse cells found in the five animals are

assumed to have resulted from damage to individual axons in plantaris at the time of injection. It may be that the diffuse and grainy cell resulted from a ventral horn cell having two (or more) branches to the region of the injection, one of which was damaged giving the diffuse stain, the other undamaged, giving the grainy stain.

3.6. The Type of Stain Resulting from Uptake by Regenerating Axons of HRP Injected into Plantaris Denervated by Section of the Sciatic Nerve in the Thigh of Juveniles

It was the object of this experiment to test whether growing axons take up HRP in a manner to cause diffuse cells. Seventeen animals received plantaris injections at varying intervals after sciatic nerve section. Both sides were injected, the right with sciatic nerve intact, serving as a control. The results are shown in Table 3.1. On the control side grainy cells appearing to conform to the normal plantaris distribution (Fig. 8.4) were found in all animals. In a few animals, one or two diffuse cells were occasionally found. These were assumed to result from damage to individual axons within plantaris by the injection.

On the experimental side the earliest sign of uptake was seen in animals injected eight days after nerve section. This consisted of rather sparse numbers of grains in a few cells. By ten days, many cells were labelled and the grain density was much more pronounced but still not as dense as the control side. By fourteen

Time of injection in days after nerve section (Each figure = one animal)	Label in ventral horn cells	
	Diffuse	Grainy
0	-	-
0	-	-
0	-	-
4	-	-
4	-	-
4	-	-
8	-	±
8	-	-
8	-	±
10	-	+
10	-	+
12	-	+
12	-	+
14	-	+
14	-	+
27	-	+
27	-	+

Table 3.1

The presence (+) or absence (-) of each type of label in ventral horn cells 44 hours after injection of HRP into plantaris denervated by section of the sciatic nerve the indicated number of days previously and allowing normal regeneration.

days, right and left sides were indistinguishable. Of those animals with grainy cells a few had one or two diffuse cells but no more than on the control side.

Since the number of diffuse cells found on the experimental sides was very few and no more than on the control sides, it may be concluded that growing nerve endings do not take up HRP in a manner to cause diffuse cells.

From these results, the growth rate of the regenerating fibres can be calculated using a distance of 0.5 to 0.8 cm between the site of sciatic nerve section and the injection site. This is calculated at 0.6 to 1.0 mm per day if eight days is taken as the time for recommencement of uptake. Certain assumptions have been made in arriving at this figure:

1. That regeneration begins immediately after section.
2. That uptake begins immediately on fibres reaching the injection site.
3. That uptake occurs only at the time of injection and not for up to 44 hours later. The first two assumptions may lead to an underestimation and the third to an overestimation of the growth rate. The calculated figure can probably be regarded as a reasonable approximation to the true figure.

3.7 Control to Assess the Importance of the Axon Shaft in HRP Uptake

Horseradish peroxidase was infused in the region of the left 8th, 9th and 10th spinal nerves where they circumnavigate the notochord. Three animals of Stage 52 were used. They were fixed 44 hours after the injection.

All three spinal cords showed diffuse and grainy primary motor neurones on the left side. One animal had no labelled ventral horn cells. Of the other two one had moderate numbers of diffuse cells and five grainy cells, the other had fewer diffuse cells and two grainy cells. The presence of moderate numbers of diffusely stained ventral horn cells and primary motor neurones was taken to indicate both damage to the spinal nerve or nerves and the presence of HRP around the nerve. The nerves cannot have been transected or badly damaged as this would have resulted in a high proportion of diffuse cells. In two animals therefore ideal conditions were obtained for testing the ability of axon shafts to take up HRP in grainy form. That is, the spinal nerves were immersed in HRP and the perineurium must have been damaged allowing HRP into direct contact with many axon shafts as evidenced by the diffuse cells, and many of the axon shafts must have been still intact. The presence of grainy primary motor neurones is in keeping with the uptake of HRP by primary motor neurone endings from the axial muscle into which the HRP was injected.

The third animal with labelled primary motor neurones but not ventral horn cells must have received an injection which involved axial musculature but not the

spinal nerves.

The two animals in which ideal conditions were achieved both had a few grainy cells (five and two respectively). It may be that these are ventral horn cells whose axon tips were growing through the part of the spinal nerve involved by the injection at the time of the injection or just afterwards. This is a reasonable assumption in view of the known ability of free nerve endings to take up materials in vitro (Hughes, 1953; Bunge, 1973). However, since such uptake has not been demonstrated conclusively in vivo, it is necessary to consider the possibility that a few axon shafts are able to take up grainy material. However, in this case, the number involved is so few, they may be ignored when compared with the number of grainy cells seen after injection into the limb.

The possibility that small amounts of HRP reached the limb by tracking or diffusion during or after the injection resulting in the few grainy cells was excluded by direct examination of the injection site. In no case was there evidence of HRP in or even close to the limb.

3.8 Discussion and Conclusions

It is concluded that axonal damage results in diffuse staining of ventral horn cells. This is in agreement with other workers (Becker *et al.*, 1968; Turner and Harris, 1974; LaVail and LaVail, 1974). It is possible that another cause of diffuse staining is histogenetic degeneration of ventral horn cells which happen to have

an axon in the injection site.

However since cell degeneration does not begin until Stage 53 it is unlikely to be a cause of diffuse staining at earlier stages unless the changes leading to degeneration begin long before actual degeneration. The large numbers of degenerating diffusely stained cells seen in many Stage 53, 54 and 55 animals may simply reflect the axotomy of Phase II cells resulting in both diffuse staining and immediate degeneration (Prestige, 1967, 1970) and does not necessarily imply that cells degenerating for purely physiological reasons stain diffusely.

Whether or not degenerating cells do stain diffusely, the fact that diffuse cells can result from damage anywhere along the axon in the presence of HRP precludes the use of diffuse cells for determining patterns of projection. However, the conclusion that the diffuse cell had an axon either ending in or passing through the site of injection is valid. This latter conclusion has been of use in determining the timing of innervation to the limb (Lamb, 1974; and Chapter 3).

In contrast to the diffuse form of uptake, the grainy form of uptake does not take place to a significant degree from the axon shafts of Stage 52 ventral horn cells at least outside the limb. It might be argued that axon shafts within the limb are able to take up HRP since they are not contained within a perineurium (Prestige and Wilson - personal communication). Apart from the

reasoning given earlier that the perineurium was breached during the injections around the notochord, thereby removing its effect, this possibility is thought not to be the case for the following additional reason.

The distribution of diffuse cells can be very different from the distribution of grainy cells in the same animal even to the point where there is very little overlap of the two distributions (Fig. 3.4). The implication is that an injection into one region involved a nerve trunk to another region as shown by the presence of diffuse cells in a different distribution to the grainy cells. Moreover, involvement of axon shafts in the nerve trunk did not lead to grainy cells in the diffuse cell distribution area; therefore axon shafts in the limb do not take up material in a grainy form.

With older animals, this question is of no practical importance however, since any axon shafts passing through the site of injection would have to have been going to the same limb region in any case, such was the way in which the limb regions for study were chosen. That is, there was no danger of injections into the chosen regions involving nerve trunks to other regions provided the injections were properly confined.

In the stage at which the possibility of axon shaft uptake of grainy material was tested and not proved, most ventral horn cells are in Phase I and very immature but their axons appear to resemble more mature nerve fibres in taking up significant amounts of HRP only from the region of their

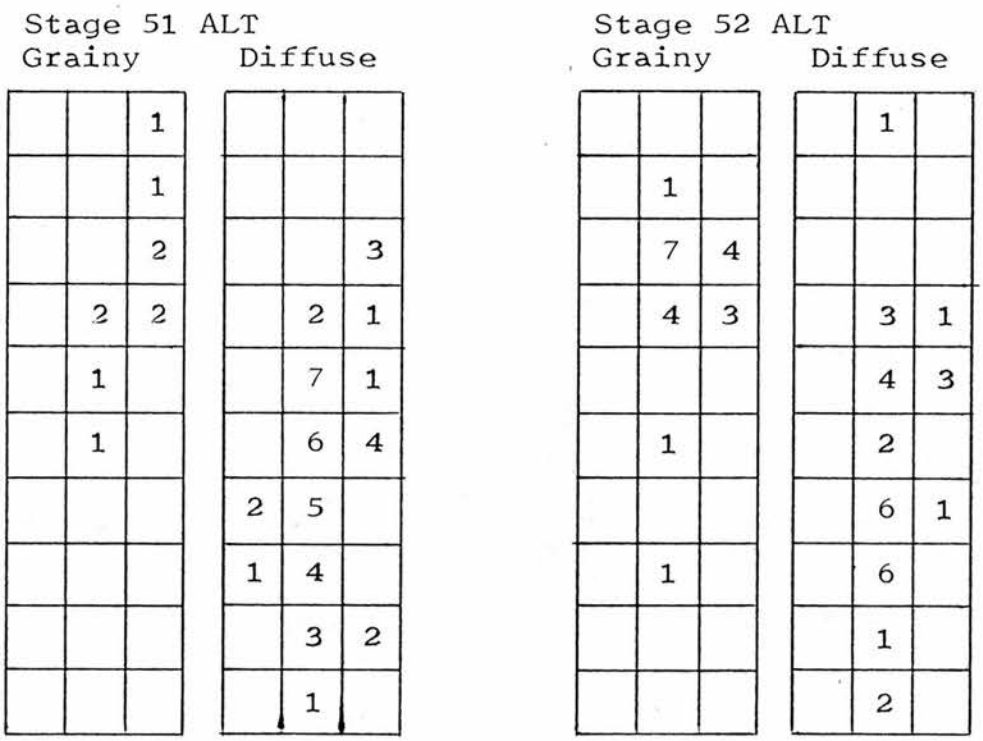


Fig. 3.4

Comparison of individual cell distribution grids for grainy and diffuse cells in two animals which received injections into the ALT. Note that each pair of grids shows little overlap.

endings. Injections into small parts of the chick optic tectum resulted in labelling of retinal ganglion cells which terminate in that region but not of retinal ganglion cells whose axons passed through the injection site to terminate elsewhere (LaVail et al., 1973). Complementary to this study, an EM study of retinal ganglion cell axons under the same experimental conditions showed many labelled vesicles apparently being formed in the region of the pre-terminal branches, but nearer the cell body, only extremely few (LaVail and LaVail, 1974). This suggests that though uptake along the axon shaft may take place as found by other workers (Holtzman and Peterson, 1969; Krishnan and Singer, 1973) either the HRP taken up is not transported centripetally or else it is in quantities too small to be detected in the cell body by standard techniques using the light microscope.

There is no direct proof that free axon endings take up HRP in granular form. However it is likely that they do for the following reasons:

1. The tissue culture evidence cited in 3.1 (Hughes, 1953; Bunge, 1973).

2. The few grainy cells found after the injections around the notochord are more easily explained by postulating that all growing endings take up material but only a few were passing through the injection site while HRP was still available than by postulating that only exceptional axon shafts have the ability to take up HRP.

3. EM studies of NMJ formation in chick limb muscle

(Hirano, 1967) and rat intercostal muscle (Terravainen, 1968; Kelly and Zachs, 1969) suggest that prior to NMJ formation, endings exist only as free endings.

Although it was argued (3.2.2) that the route of transport of HRP to the labelled ventral horn cells was intra-axonal, this still leaves unanswered the question of whether all labelled cells have endings in the site of the injection. It is theoretically possible that some labelled cells obtain their HRP by inter-cellular transfer either via the axons or the dendrites. Tight junctions have been described between multiple axons within a common myelin sheath in adult mouse sciatic nerve (Waxman, 1968) though there is no evidence of material transfer through these. Similar tight junctions have not been found in Xenopus hind limb nerves (Prestige and Wilson - personal communication). The only direct evidence of material transfer between ventral horn cells is from a study in which procion yellow, a low molecular weight dye was iontophoretically infused into single ventral horn cell bodies of cat lumbar spinal cords (Zieglgansberger and Reiter, 1974). In about half the cases, one and sometimes two neighbouring but not necessarily juxtaposed ventral horn cells became labelled. The authors suggested that the transfer took place between dendrites though they provided no evidence. As the technique necessitates damage to at least one cell membrane it is highly questionable whether this process takes place in the normal animal. Indeed the photographic evidence shows a diffuse stain also of the secondarily

stained cells which suggests they were damaged too. Even if such a process does take place normally with small molecules, it then has to be shown to occur with the very much larger HRP molecule which is nearly two orders of magnitude heavier than procion yellow.

Even if local transfer of HRP does occur in the same way, then the short range of transfer can have no effect on the rostro-caudal distribution of labelled cells. With respect to the medio-lateral axis, it is clear from the results of Chapters 6 and 7, which show clearly demarcated medial and lateral distributions of labelled cells for different injection regions, that either inter-cellular transfer does not occur medio-laterally, or at most only to immediately adjacent cells and no more. This is so for animals of Stage 53 and more. Below Stage 53 such transfer would be of no importance since there is in effect no medio-lateral sub-division in terms of innervation patterns because no prospective adult lateral cells so far exist (see 1.2.1).

Even in the study with procion yellow, no transfer was found between widely separated ventral horn cells so it is likely that the much larger HRP molecule is not widely transferred and it was assumed that this is the case.

It was finally concluded that though the faint possibility existed that some grainy cells may have become labelled by a route other than the retrograde axonal route from the injection site these would have had no significant influence on the <u>patterns</u> of distribution

of labelled cells innervating various limb regions.

The mapping of grainy cells was therefore considered a valid method of determining the regions of the ventral horn which innervate specified limb regions at successive stages of development.

CHAPTER 4

A DESCRIPTION OF DIFFUSELY LABELLED CELLS

4.1 Introduction

Apart from red blood cells and, in the special case described in 3.7, primary motor neurones, it is presumed that all diffusely labelled cells are ventral horn cells which are diffusely labelled by virtue of their axon being damaged in the presence of HRP. This gives a positive means of identifying the youngest ventral horn cells individually. This had not previously been possible leading to uncertain criteria for their identification. The problem has been especially relevant in marginal situations such as at the ends of the ventral horn, or the medial border in the youngest animals or in the case of individual cells within the ventral horn which do not conform to the criteria of shape, orientation and size usually described, or individual cells that conform in all respects except that of position, being outside the boundaries of the ventral horn. Taken together, these variations can have a significant effect on counts of ventral horn cells especially at young stages (Hughes, 1961; Prestige, 1970; Kollros, 1968).

In the following descriptions, the emphasis is therefore on the extremes of shape, size, orientation and position that are seen, though because dendrites are displayed so clearly a description of these is included.

The descriptions are from many animals of each stage after HRP injections into all the limb regions described in the various experiments of this thesis.

4.2 Observations

4.2.1 Stage 50

The ventral horn is just distinguishable as small numbers of cells tending to align circumferentially at the lateral edge of the mantle layer over a dorso-ventral width about equal to the dorso-ventral height of the central canal. The rostral and caudal ends are very indistinct and appear to fall short of the extent seen in Stage 51. This is especially so at the caudal end which seems to lie only a short distance caudal to the entrance of 10th dorsal root.

Few diffuse cells are seen at this stage and nearly all are at the medial boundary of the ventral horn as judged by the fact that only radially arranged cells, presumed to belong to the mantle layer are seen more medially. However, occasionally, diffuse cells are seen which appear to jut out of the mantle layer and are oriented between radial and circumferential (Fig. 4.2 A). These are usually seen at the ventral margin of the ventral horn. Labelled cells are frequently seen very dorsally, even dorsal to the level of the top of the canal. No diffuse cells were seen near or beyond the ends of the ventral horn as determined by other criteria (2.10.2, 2.10.7). All cells were about the same size (longest diameter 8 μm) and varied from oval through semicircular to spindle shaped. The nucleus occupied nearly the entire perikaryon and was generally responsible for the shape of the cell. Many cells had one or two short processes visible usually at the dorsal and

ventral poles. These were never seen extending beyond the boundaries of the ventral horn, though the axon must have reached the limb. The dorsal dendrite was frequently branched into two (Fig. 4.1 A).

4.2.2 Stage 51

The ventral horn is now a clearly visible structure of many circumferentially oriented cells forming a distinct hump bulging out from the mantle layer. The central canal is considerably higher than in Stage 50 resulting in the ventral horn appearing to lie more ventrally and not extending as far dorsally. The rostral end is much more distinguishable with a fairly sharp boundary over about 50 - 80 μm. The caudal end is still indistinct but definitely lies well caudal to the entrance point of the 10th dorsal root.

Diffuse cells can be present in large numbers and may lie anywhere in the ventral horn. However a few are seen ventral to the ventral horn and lying in a radial orientation (Fig. 4.2 B). A greater number are found dorsal to the ventral horn, some about the level of the top of the canal (Fig. 4.2 C). Diffuse cells are rare at or beyond the ends of the ventral horn as determined by other criteria. One was found in each of two animals within a couple of cross sections beyond the apparent caudal end. However, occasional grainy cells were frequently visible for up to sixty or eighty micrometers beyond the apparent caudal end. At the rostral end, grainy cells were sometimes found up to fifty micrometers beyond the apparent end.

Fig. 4.1

Diffusely stained ventral horn cells at successive stages to show stages of dendritic development. A to D all transverse sections. All sections from animals killed 44 hours after HRP was injected into various parts of the limb. Sections prepared as described on p. 37 et seq.

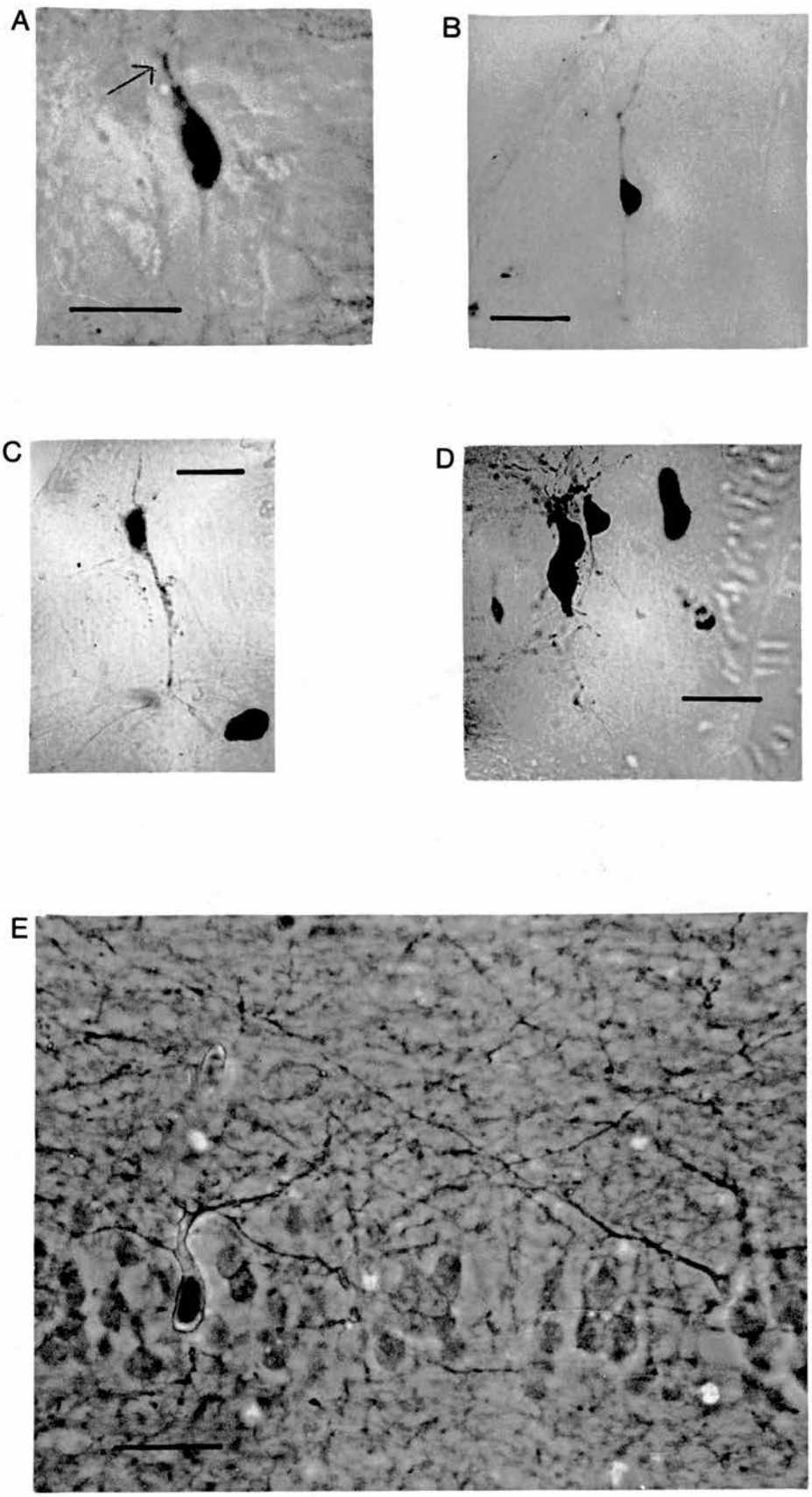
A: Stage 50. Note forked dorsal dendrite (arrow) which is not visible beyond the boundaries of the ventral horn. Scale = 10 μm.

B: Stage 51. Dorsal dendrite clearly visible extending into dorsal white matter. Scale = 20 μm.

C: Stage 52. Numerous branched dendrites extending to dorsal, lateral and ventral white matter. Fibre leaving bottom left of photograph may be the axon. Scale = 20 μm.

D: Stage 53. Dendritic branching increased further. Note dendrite extending towards ventral commissure (bottom centre). Scale = 20 μm.

E: Stage 53. Longitudinal section to show longitudinal extent of dorsal dendritic ramification. Ventral dendrites are equally ramified. (Phase contrast). Scale = 30 μm.


A
B
C
D
E

Most diffuse cells were circumferentially orientated though some as mentioned above may lie radially both within and outside the ventral horn. Though most cells are no larger than in Stage 50 (8 μm long diameter) a few are larger (up to 12 μm long diameter). Cell shapes are also more varied though a rather round spindle shape is still the most common. Other cells are round, roughly triangular or polygonal and the nucleus still occupies virtually the entire perikaryon. Most cells have at least a dorsal and a ventral process. The ventral process is sometimes branched and the dorsal process usually is and often into several branches. Both dorsal and ventral processes are visible beyond the ventral horn. The dorsal sends branches to the dorsal white matter and the lateral subpial white matter (Fig. 4.1 B) though sometimes this branch springs directly from the cell body (Fig. 4.1 C). The ventral process sends a branch which may be the axon towards the ventral root and sometimes a branch in the direction of the ventral commissure.

4.2.3 Stage 52

Both ends of the ventral horn are now readily distinguishable. Though no diffuse cells have been seen beyond these ends, a few individual grainy cells may be seen up to about 70 μm beyond either end.

Diffuse cells are less frequently seen lying outside the ventral horn dorsally or ventrally although the occasional radially orientated cell is still seen. Some

Fig. 4.2

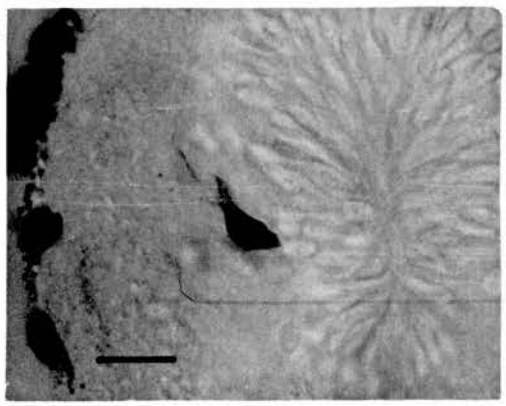
Variations of position and orientation of diffusely labelled ventral horn cells. All transverse sections. All sections from animals killed 44 hours after HRP was injected into various parts of the limb. Sections prepared as described on p. 37 et seq.

A: Stage 50. Ventral horn cell still partly within the mantle layer with semi-horizontal orientation. Note the short dorsal dendrites. Scale = 10 μm.

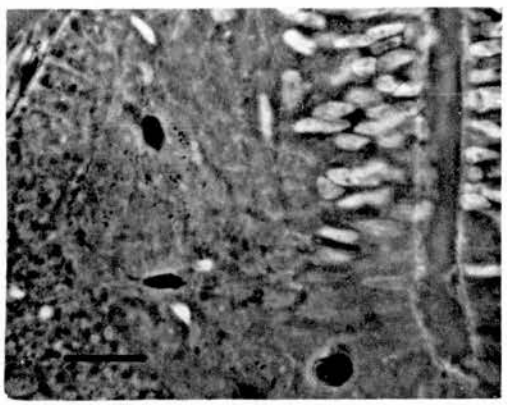
B: Stage 51. Horizontally orientated ventral horn cell at ventral border of the ventral horn. Normally oriented ventral horn cell is visible above. (Phase contrast). Scale = 20 μm.

C: Stage 51. Ventral horn cell of normal circumferential orientation but lying above the dorsal border of the ventral horn. (Phase contrast). Scale = 10 μm.

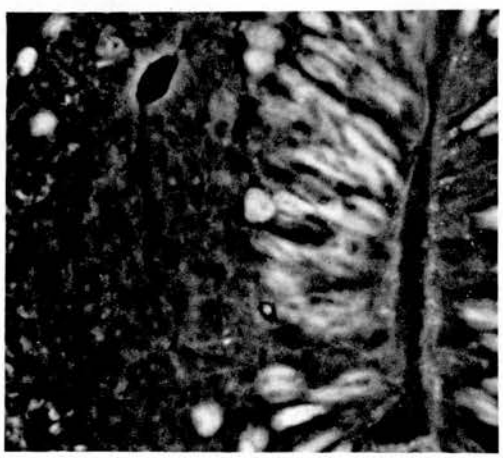
A



B



C



cells are now quite large (up to 16 μm) and these have profuse dendritic branching at both ends (Fig. 4.1 C). In contrast to diffuse cells at earlier stages, the largest cells in Stage 52 may look unhealthy with varicosities of the dendrites and often a ragged looking membrane.

4.2.4 Stage 53

At this stage, diffuse cells are very rarely seen outside the ventral horn and then only dorsally. All cells are circumferentially orientated. Occasional individual grainy cells are seen beyond the apparent ends both rostrally and caudally. Many diffuse cells up to 15 to 18 μm are seen though smaller cells resembling those described for earlier stages are also seen. Apart from an increase in the size of the cytoplasmic caps at the poles, the nucleus still occupies most of the perikaryon. Many of the larger cells look unhealthy as described above and some are frankly degenerating (Fig. 3.2 B).

In addition to the dorsal and ventral dendrites which are now profusely branched to the areas already described at Stage 51 many cells have further dendrites growing from the cell body. A common feature by now is a dendrite growing to the ventral commissure though none have been traced as far as the opposite side (Fig. 4.1 D). Dendrites at Stage 53 have also grown considerable distances along the rostro-caudal axis of the spinal cord (Fig. 4.1 E).

4.2.5 Stages 54 and 55

Smaller numbers of diffuse cells are now seen probably because nerve trunks are less frequently damaged during injections. No diffuse cells larger than those in Stage 53 were ever seen and except for small, apparently immature cells all of these were degenerating. In addition no increase in the amount of cytoplasm was seen in diffusely stained cells. Dendrites were broken into short varicose segments but the general layout resembled that seen in Stage 53.

A feature at this stage was significant numbers of cells with a pale diffusely labelled nucleus but otherwise unlabelled and healthy. These cells were frequently larger than diffuse cells and had a higher cytoplasm to nucleus ratio.

4.2.6 Stages 58, 59 and juvenile

Healthy looking diffuse cells were occasionally seen in these stages. They were identical in every way with normal juvenile cells except that the cells in younger animals were a little larger.

In the series described in 3.4 large numbers of diffuse cells were seen. Many of these looked unhealthy with varicosities of the dendrites, ragged cell membranes and eccentric nuclei (Fig. 3.3 C).

4.3 Discussion

4.3.1 Ventral horn cell identification

The central objection to these descriptions is that the cells described have had their axons damaged. This is almost certainly the explanation of the failure to see healthy diffuse cells beyond a certain level of development until about Stage 58. Cells with damaged axons can only survive the 44 hours if they are in Phase I or have been in Phase III for a few stages (Prestige, 1967, 1970). Since all cells in Stages 53, 54 and 55, if not small and resembling earlier stage cell types, are post Phase I, but only just, they are degenerating by the time of examination 44 hours later because they have been axotomised. Some cells had only faintly labelled nuclei and looked larger and healthy. These may have been cells with only minimal trauma to the axon permitting survival.

Whether the anatomy of the dendrites of Phase I cells is altered by axonal damage or HRP is not known.

A further objection in these descriptions is that by definition all diffuse cells must have an axon in the limb. Characteristics of diffuse cells may therefore not apply to cells which have not grown an axon into the limb. Though the proportion of the latter is small at any stage since all ventral horn cells grow their axon to the limb almost immediately it still forms a significant proportion of the total cell count so that the objection could be important.

However, it is unlikely that these descriptions will

lead to over-inclusion of cell morphologies in ventral horn cell designation and counting. If any error can occur, it will be that certain variances from the standard criteria for identifying young ventral horn cells will not be detected by this method. However, the method has sharpened the criteria:

1. No cells medial to the most medial circumferentially orientated cells in young animals or medial to the large medial ventral horn cells in Stages 53 onwards should be counted. The latter of these points is of particular relevance because at about Stage 54, cells medial to the large ventral horn cells develop into small neurones which might be mistaken for ventral horn cells. However these were never seen to become labelled.

2. Cells dorsal to the ventral horn but which otherwise resemble ventral horn cells may be counted at Stages 50, 51 and 52.

3. Radially orientated cells within or ventral to the ventral horn may be counted if they otherwise resemble ventral horn cells at Stages 50, 51 and 52.

4. The standard criteria for identifying the ends of the ventral horn, that is circumferentially oriented cells lying at the lateral edge of the mantle layer may be used with greater confidence. A few individual cells are found beyond the boundaries as established by standard criteria at all stages including the juvenile but the total number is small.

4.3.2 Dendritic Growth

At Stage 50 ventral horn cells have only very short dendrites which are frequently visible as only a short spike. It has been suggested that ventral horn cells grow their dendrites only after contacting the limb (Barron, 1943; Jacobson, 1970). Though because of the nature of the experiment, the cells described here must have contacted the limb, the presence of such short dendrites does support this idea. However dendritic growth beginning earlier cannot be ruled out.

The dendritic descriptions given are only of dendrites within the plane of single sections which probably results in an underestimation of the degree and extent of dendritic growth. This may also result if fine dendrites are below the resolution of the light microscope. Nevertheless, it is with reasonable confidence that the following conclusions are made.

1. Dendritic growth begins very early in ventral horn cell development around the time that they first contact the limb.

2. No growth of dendrites occurs beyond the boundaries of the ventral horn until Stage 51. At Stage 51 all the main dendritic branches are visible on some cells and perhaps exist on most.

3. By Stage 53 dendrites have expanded to all areas seen in the later stages except possibly for the dendrite crossing to the opposite side in the ventral commissure. None were seen on the other side at Stage 53 although they were growing in that direction. Such dendrites were seen in Stage 58 and 59 animals but not in juveniles.

CHAPTER 5

THE TIMING OF THE EARLIEST MOTOR INNERVATION TO THE DEVELOPING LIMB BUD

5.1 Introduction

In this chapter, the fact that the presence of labelled ventral horn cells in the ventral horn indicates the presence of their axons in the site of injection of HRP has been used to determine when the limb first becomes innervated. Ventral horns were examined for labelled cells after injection of HRP into the limb in successively older tadpoles. The stage at which labelled ventral horn cells were first seen is the stage when ventral horn cell innervation to the limb is presumed to begin, subject to certain assumptions discussed at the end of the chapter.

Since it was also not known whether innervation of different limb regions begins simultaneously the same principle of investigation was applied to specified individual regions of the limb. The results of the latter investigations are presented in 5.3.

5.2 The earliest motor innervation to the limb bud as a whole

Five batches of animals plus two animals from a batch of which the rest died after the injection (asterisks) were used.

A large injection was given into the proximal part of the left limb or the whole limb in the youngest cases. The volume of HRP injected was estimated to be from about 0.02 µl in the youngest animals to about 0.2 µl for the

oldest. A total of 39 animals was examined for labelled ventral horn cells.

Results

The results are presented in Table 5.1.

The earliest stage at which uptake was seen is Stage 50 when four out of eight animals had labelled cells in the left ventral horn. Of these, two had grainy cells only and two had grainy and diffuse cells. In one of the former cases, grainy material was sparse but definitely present.

From Stage 51 onwards, all animals examined had grainy cells and most animals had diffuse cells. However some animals of Stages 51 and 52 had no diffuse cells.

Diffuse cells were never present alone.

There was no difference in the time of first appearance of grainy and diffuse cells. However, in addition to diffuse cells being seen less reliably, they were often present in only small numbers at all stages. Conversely, some animals at all stages except Stage 50 had large numbers of diffuse cells, there being no correlation of diffuse cell numbers with stage.

These results are discussed together with the results of 5.3 at the end of the chapter.

Stage (Each figure = 1 animal)	Body length (mm)	Leg length (mm)	Shape of leg	Diffuse Cells	Grainy Cells
49	19	0.15	Cr	-	-
49	20	0.2	Cr	-	-
49	20	0.25	Cr-R	-	-
49	22	0.3	Cr-R	-	-
49	22	0.3	Cr-R	-	-
49	22	0.3	Cr-R	-	-
49	22	0.33	Cr-R	-	-
49	24	0.4	Cr-R	-	-
50	23	0.4	R	-	-
50	23	0.4	R	+	+
50	24	0.4	R	-	-
50	22	0.5	R	+	+
50	24	0.5	R	-	-
50	24	0.5	R	-	-
50	24	0.5	R	-	+
50	25	0.5	R	-	+
51	23	0.6	R-C	-	+
51	26	0.6	R-C	-	+
51	26	0.6	C	+	+
51	25	0.7	C	+	+
51	25	0.7	C	+	+
51	23	0.8	C	+	+
*51	25	0.8	C	+	+
51	27	0.85	C	-	+
51	27	0.9	C	-	+
*51	24	1.0	C-P	+	+
51	30	1.0	C-P	-	+
52	30	1.1	C-P	-	+
52	33	1.1	C-P	+	+
52	34	1.3	P	+	+
52	39	1.3	P	+	+
53	35	1.4	P-T	+	+
53	39	1.4	P-T	+	+
53	35	1.5	P-T	+	+
53	36	1.6	P-T	+	+
53	37	1.7	P-T	+	+
54	37	1.9	T	+	+
54	42	2.7	T	+	+
54	40	2.8	T	+	+

Table 5.1

Presence (+) or absence (-) of each type of label 44 hours after injection. Animals staged at time of fixation. Cr: crescent shaped. R: round. C: conical. P: paddle. T: toes differentiating. Asterisks indicate two survivors of a batch of which the rest died after injection.

5.3 The earliest motor innervation to individual limb regions

The results of this part were obtained from the experiments of Chapters 6 (Juvenile injections) and 7 (Larval injections) where details of injection volumes are given.

Injections of HRP small enough to be confined to the following regions were given:

1. The antero-lateral aspect of the thigh (ALT)*
2. The postero-medial aspect of the thigh (PMT)*
3. The distal tip of the limb bud (distal)*

From Stage 52 onwards, attempts were made to confine distal injections to either the dorsal (ALA)* or the plantar side (plantar)* of the foot pad and ankle region. Those that succeeded are presented separately. Those that failed (i.e. where injection involved both compartments) are counted as a separate group (Mixed)*.

Success or failure of an injection for both proximal and distal regions was assessed by examining the limb bud histologically for the extent of the injection site (Figs. 3.1 A-D). In proximal injections, failure meant that the injection site involved areas other than the intended area and these were rejected. In distal injections failures were of two types:

1. Mixed - these were examined.
2. Injections involving areas proximal to either the tip of the limb bud in the Stage 50 or 51 animals, or the foot and ankle region in those animals of Stage 52 or more. These were rejected.

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* These abbreviations are used constantly throughout the thesis and are summarised and explained in Fig. 1.1.

Stage	Number injected	Rejected	+	-	%+
49	1	0	0	1	0
50	15	5	5	5	50
51	21	4	14	3	84
52	14	2	11	1	92
53	13	1	11	1	92
54	10	0	10	0	100
55	6	0	6	0	100
58	4	0	4	0	100
J	6	0	5	1	85

Table 5.2

Number of animals receiving injections into ALT and numbers of positive (grainy)(+) and negative (-) results and percentage of positive from total (%+) for each stage. Injections involving other regions are rejected. J: juvenile.

Stage	Number injected	Rejected	+	-	%+
50	13	6	3	4	43
51	19	11	8	0	100
52	13	3	10	0	100
53	8	1	7	0	100
54	10	0	10	0	100
55	4	0	4	0	100
58	5	0	5	0	100
J	10	0	10	0	100

Table 5.3

Similar tabulations for injections into PMT.

Results

Only grainy cells were considered as positive evidence of innervation of the limb region in question. However in no region did diffuse cells appear earlier than grainy cells.

The results are presented to show both the timing of the earliest innervation to the specified region and the percentage of positive results for each region at each stage. (Tables 5.2, 5.3, 5.4).

There is no difference of timing of the earliest innervation to the two thigh regions, which begins in Stage 50 and is firmly established in Stage 51 in both regions. However, whereas all injections into the PMT resulted in labelled cells from Stage 51 onwards, a small proportion of animals with ALT injections showed no uptake at most stages including juvenile.

There is a very clear difference in timing of earliest innervation to proximal and distal regions. The earliest uptake from the foot and ankle regions is seen at Stage 52 or two stages after proximal innervation.

There is no difference in the timing of innervation to the ALA and plantar region. However there is a tendency for there to be some negative results in the ALA series at later stages.

5.4 Discussion and Conclusion

It is concluded that the first ventral horn cells innervate the limb at Stage 50. At this stage the limb is

Stage	Number injected	Rejected	Plantar region			ALA			Mixed		
			+	-	%+	+	-	%+	+	-	%+
50	4	0	-	-	-	-	-	-	0	4	0
51	25	2	-	-	-	-	-	-	0	23	0
52	28	0	3	3	50	2	6	25	5	9	35
53	14	0	2	0	100	7	1	88	4	0	100
54	18	0	7	0	100	3	0	100	8	0	100
56	15	0	5	0	100	5	0	100	5	0	100
J	22	9*	7	0	100	5	1	85	-	-	-

Table 5.4 Numbers of animals injected at each stage with numbers of positive (+) and negative (-) and percentage of positive results from total (%+) for injections confined entirely to plantar region and ALA at each stage. Injections involving both compartments are classed as mixed. Injections involving regions proximal to the correct regions are rejected. J = juvenile.

* See text.

very immature and composed of rapidly dividing cells from which muscle has yet to begin differentiating. Indeed myotube formation and hence NMJ formation does not begin until two stages later. However, the very young axons are capable of mediating functional neuromuscular transmission if given sufficiently mature muscle to innervate (Letinsky, 1974). The anatomical structure of the axon endings and their relationship to the mesenchymal cells are unknown though an E.M. study in chick suggests that endings in early limb neuromotor development form no specialized contacts (Hirano, 1967). The function, if any, of such early innervation is unknown. Neither is it known whether any interaction takes place between the nerve fibres and the mesenchymal cells at this stage in the normal animal.

The whole limb bud is not innervated simultaneously. Distal regions of the limb bud are innervated considerably later than proximal regions with a delay of two stages. This is not simply due to the fact that distal regions do not develop until later: up until Stage 52, there is always a region distally that is uninnervated. Since this is the youngest part of the limb, it appears that the tissues will only permit nerve fibre entry when they have reached a certain stage of maturity. This may correspond with the time at which the tissue leaves the progress zone. The progress zone has been calculated to be 230 $\pm$ 70 μm thick in the chick (Wolpert et al., 1975). This represents about half the length of a Stage 50 limb and a third the length of a Stage 51 limb, thus distal injections would easily have

been contained entirely within the progress zone at these stages. This idea is attractive because according to the progress zone model, cells do not possess a position dependent label until they leave the progress zone (Summerbell et al., 1973) and these labels may be required to guide axons to their synapse point.

In this discussion so far, two assumptions have been made. The first is that axon endings transport HRP as soon as they enter the limb. Although this is unlikely not to be so in view of the fact that in vitro, even free endings have been reported to take up materials (Hughes, 1953; Bunge, 1973) it would be of little significance if it were not as the ventral horn itself does not appear until Stage 50 and any delay before the commencement of uptake could not be more than half a stage. The second assumption is that failure of uptake from distal regions is due to the absence of innervation of these regions. Although the same argument about free nerve endings applies here too, it is not certain that uptake is not mediated in some way by mesenchyme cells and that this function cannot be carried out unless they are of a certain maturity. However, the strongest argument in favour of both these assumptions is that diffuse cells never resulted either with pre-stage 50 injections or with distal injections at Stages 50 and 51. Since diffuse cells represent axonal damage, it is difficult to see why axonal damage at the distal end of the axon should produce different results from that occurring anywhere else

including a region only 300 μm more proximally in the latter case. In view of this it is felt that making these assumptions can be justified.

These results confirm the conclusion reached by Taylor, 1943 and Hughes and Tschumi, 1958, that ventral horn innervation takes place very early in limb bud development in anura. This is in apparent contrast to a recent report that motor innervation of the chick limb takes place relatively much later in development (Hamburger, 1975). He contends that the time of innervation coincides with myoblast differentiation and ventral horn cell degeneration (6 days) although scrutiny of the report shows that innervation actually begins before this (4½ days) which conforms with the known existence of choline acetyl transferase (a specific indicator of cholinergic axonal presence) in the chick limb bud by day 3 or 4 (Giacobini et al. 1973). Hamburger also noted that innervation of the more distal regions took place only slowly over two or three days which is in basic agreement with the present results.

The appearance of ventral horn axons in the limb bud almost simultaneously with the first appearance of ventral horn cells (Prestige, 1970) and small unmyelinated ventral root fibres (Prestige and Wilson, 1972, 1974) indicates that at least at Stage 50 there is virtually no delay in axons reaching the limb bud. If the assumption is made that the same applies to all axons which reach the ventral root, then since all ventral horn cells grow an axon into

the ventral root immediately (Prestige and Wilson, 1972, 1974) these results can be used as indirect support for all ventral horn cells growing an axon almost immediately into the limb bud. This contention is virtually proved by a recent unpublished experiment (Prestige and Wilson - personal communication, see p. 11).

Of great concern at the start of the experiments requiring small localized injections was the possibility that HRP might diffuse to regions other than the intended one. The present results indicate that diffusion is a negligible problem. Injections into the distal regions of Stage 50 and 51 limb buds could not have diffused into proximal regions since then it would have been taken up by axons terminating there. Yet no uptake following distal injections at these stages was ever seen. The distance between proximal and distal regions in a Stage 50 limb bud cannot be more than about 250 μm. This must represent more than the maximum distance of HRP diffusion though the distance may be much less. This is indeed suggested by the results of Chapter 7 where injections in the foot and ankle region separated by as little as 70 to 100 μm give quite different and non-overlapping patterns of labelled cell distribution. The possibility still exists that diffusion of HRP could take place to regions proximal to the base of the limb bud affecting the results of 5.2. However uptake from individual limb regions still took place at Stage 50 and with these injections it was possible to be certain from histological examination that HRP had not diffused

outside the intended site. In addition, diffuse cells were seen at Stage 50 indicative of damage to axons at the injection site.

Sporadic negative results occurred in the ALA and ALT series. These probably resulted from failure of adequate injection. This can occur if the HRP leaks straight out along the shaft of the pipette or, in older animals if the injection fails to penetrate the connective tissue sheaths around the muscles resulting in a completely extra-muscular injection. The latter is especially likely to occur in the ALT and the dorsum of foot where muscle penetration is considerably more difficult than in other regions in older animals.

CHAPTER 6

PATTERNS OF VENTRAL HORN CELL INNERVATION TO THE HIND LIMB OF THE JUVENILE

6.1 Introduction

A description is required of the patterns of innervation in the juvenile to the four regions of the limb to be studied in larval stages. It is not the aim of this chapter to make a detailed study of the whole limb innervation comparable to the work of Cruce (1974) or Romanes (1951), and no attempt has been made to study other regions or individual muscles except in special cases where required.

Since it was desirable that as much correspondence as possible should be maintained between the region of the limb involved in larval injections and juvenile injections, limb compartments were studied as a whole in the juvenile since whole limb compartments are the limit of resolution in the youngest limb bud stages.

The four regions studied were:

1. The antero-lateral thigh (ALT)
2. The postero-medial thigh (PMT)
3. The plantar region
4. The antero-lateral ankle region (ALA).

The muscles contained in each of these compartments, and their actions are described in the discussion (6.3).

Injections of 0.1 to 0.5 µl were given into the left limb for each region. The animals were fixed and processed after 44 hours and the labelled cells in the ventral horn were mapped and grids prepared according to the procedures described in Chapter 2 (2.10, 2.12).

Only grainy cells were mapped since the preliminary experiments (Chapter 3) showed that grainy material is taken up only in the region of the axon terminations. Diffuse cells, on the other hand, may result from uptake due to damage anywhere along the axon shaft making them unsuitable for projection studies.

The limbs were dissected and treated separately in order to determine the site and extent of the injection histologically.

6.2 Results

6.2.1 Injection site

Difficulties were encountered with both thigh and distal injections. In the former, many injection sites failed to stain adequately. In most of these cases, the brown stain could be seen only in the superficial layers and the site of the injection was often completely indistinguishable. In the few cases where the injection site could be adequately determined, it was confined to the intended area. In view of the ease of giving injections confined to the intended site in juvenile thighs, it was assumed that no injections had spread beyond the intended site and therefore all animals in the proximal categories have been included in the assessment of proximal patterns.

With distal injections the main obstacle to determining the site of injection was the tendency for the non-specific brown stain to obscure the stain resulting from injected HRP. In most cases, it was not possible to define accurately, how

much of the intrinsic musculature was involved by the injection. In addition to this problem, in one group of animals in which plantar injections were given, the limbs were not kept for histology. In a further group of animals in which injections were given into the ALA, the injection sites failed to stain. In this last group, since injection into this site is easily confined, the same assumption was made as for the thigh, that all injections were adequately sited. All animals in this group are included in the assessment. The situation for injections into the foot itself however, is more complex. Injections into both plantar and dorsal aspects of the foot are difficult, especially the latter, and it could not be assumed that all injections were correctly sited. Of nine plantar injections, one was rejected since HRP could be seen histologically to have extended into the ankle region. However, this animal was mapped (Fig. 6.6). The map contained a combination of the patterns seen after injections into the ALA and the plantar region. Of the remaining eight animals, all gave consistent patterns except one. This animal also showed the combined pattern as described above. Since this animal was from the group in which the limbs had not been kept, it could not be immediately rejected on the grounds of spread of HRP to non-plantar regions. However, since the pattern conformed exactly to the combined pattern and was so at variance with the other seven animals it was not included in the plantar grids.

Injections into the dorsum of the foot are very difficult both from the point of view of impaling muscle and keeping

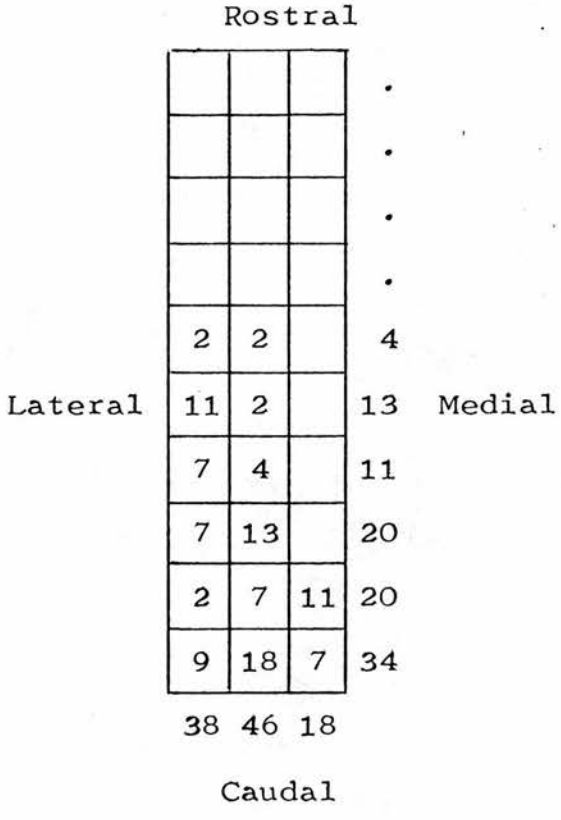


Fig. 6.6

Individual percentage distribution grid of one juvenile receiving a plantar injection which on histological examination was found to have involved the ankle region including the ALA in addition to the plantar region. A combination of the plantar pattern and the ALA pattern has resulted (combined pattern).

the injection properly confined. The large majority of animals was rejected at the time of injection as HRP was clearly seen entering the wrong area. This was usually the plantar compartment, or else HRP tracked proximally into the ALA. Of the seven animals examined, three had no labelled cells at all, three showed the combined pattern as described above and one, the pattern seen after injection into the ALA. Since the injection sites could not be determined, and in view of the highly variable results, this region was abandoned for further study and the slightly more proximal and experimentally more favourable dorsiflexor musculature in the tarsal region was used to obtain the juvenile ALA pattern.

A consideration of the development of the limb justifies the assumption that the designated ALA in the juvenile is homologous with the designated ALA of the Stage 52 to 56 limb buds. The elongation of the tarsal region to form a region separable from the back of the foot by the present technique does not occur until after Stage 56 (Nieuwkoop and Faber, 1967).

6.2.2 Innervation pattern to the ALT (Fig. 6.1)

The largest proportion of ventral horn cells innervating the ALT lies in the caudal half of the ventral horn though mainly rostral to the most caudal fifth. A smaller proportion of cells lies rostrally and a few ventral horn cells of even the most rostral end of the ventral horn may send axons to this region (animals A and E in Fig. 6.1 B). There is a strong lateral distribution at all levels.

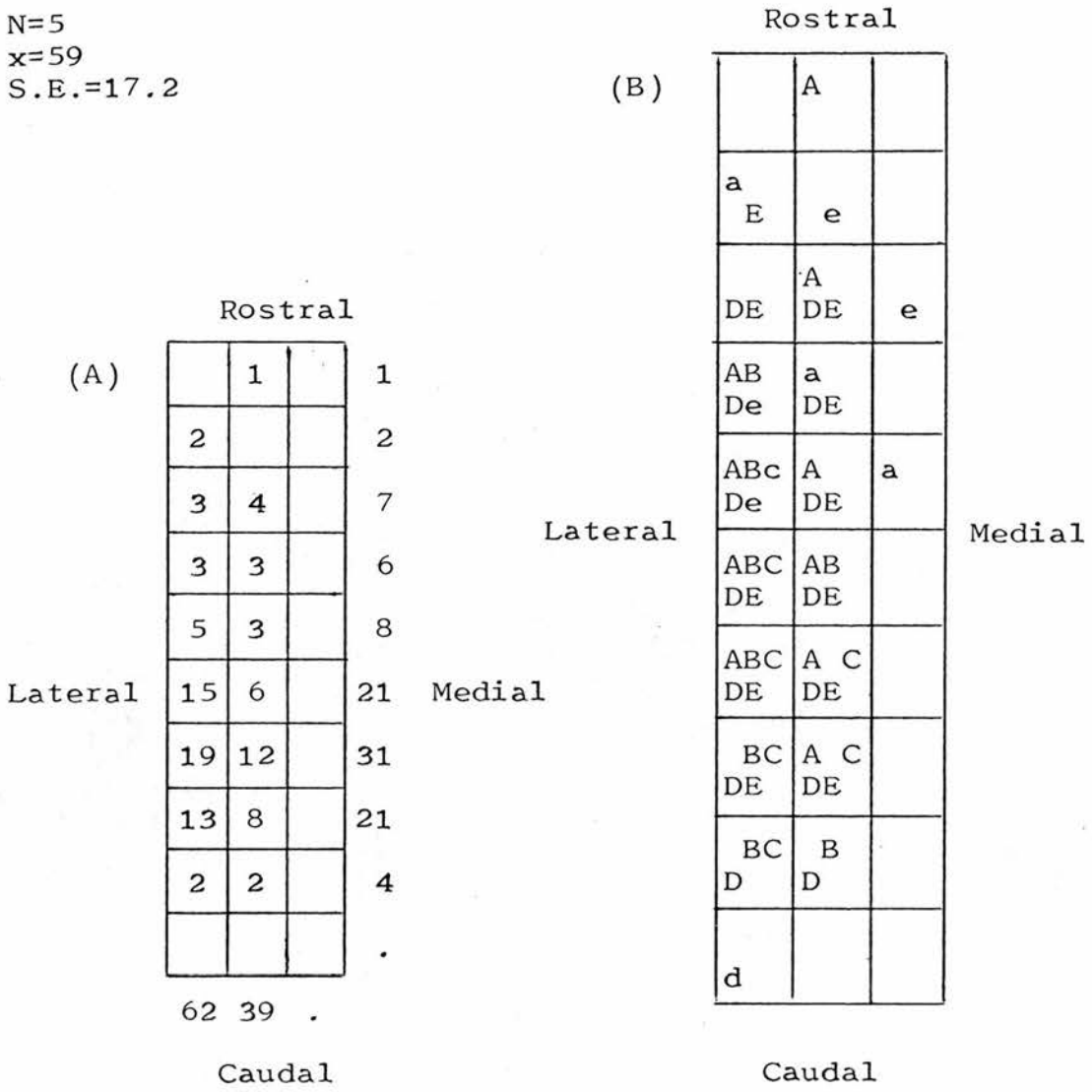


Fig. 6.1

Averaged percentage distribution grid (A) and distribution variation grid (B) for labelled cells resulting from injections into the ALT of juveniles. N: number of individual percentage distribution grids in the group. x: average number of labelled cells per animal in the group. S.E.: standard error. Note that N may be less than the number of animals in the group (see Fig. 2.2). x and S.E. are calculated from the total number of animals with labelled cells not N.

N=9
x=113
S.E.=28.1

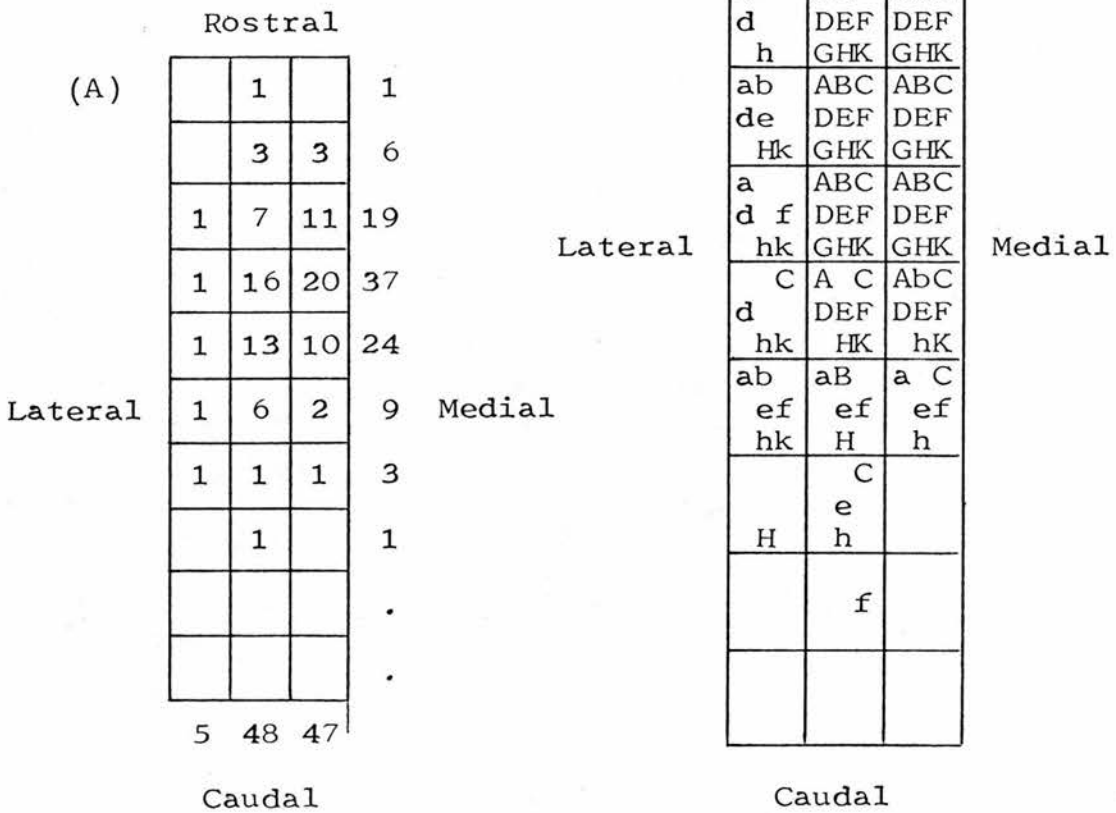


Fig. 6.2

Grids for PMT injections in the juvenile (Conventions as for Fig. 6.1).

N=7
x=30
S.E.=11.5

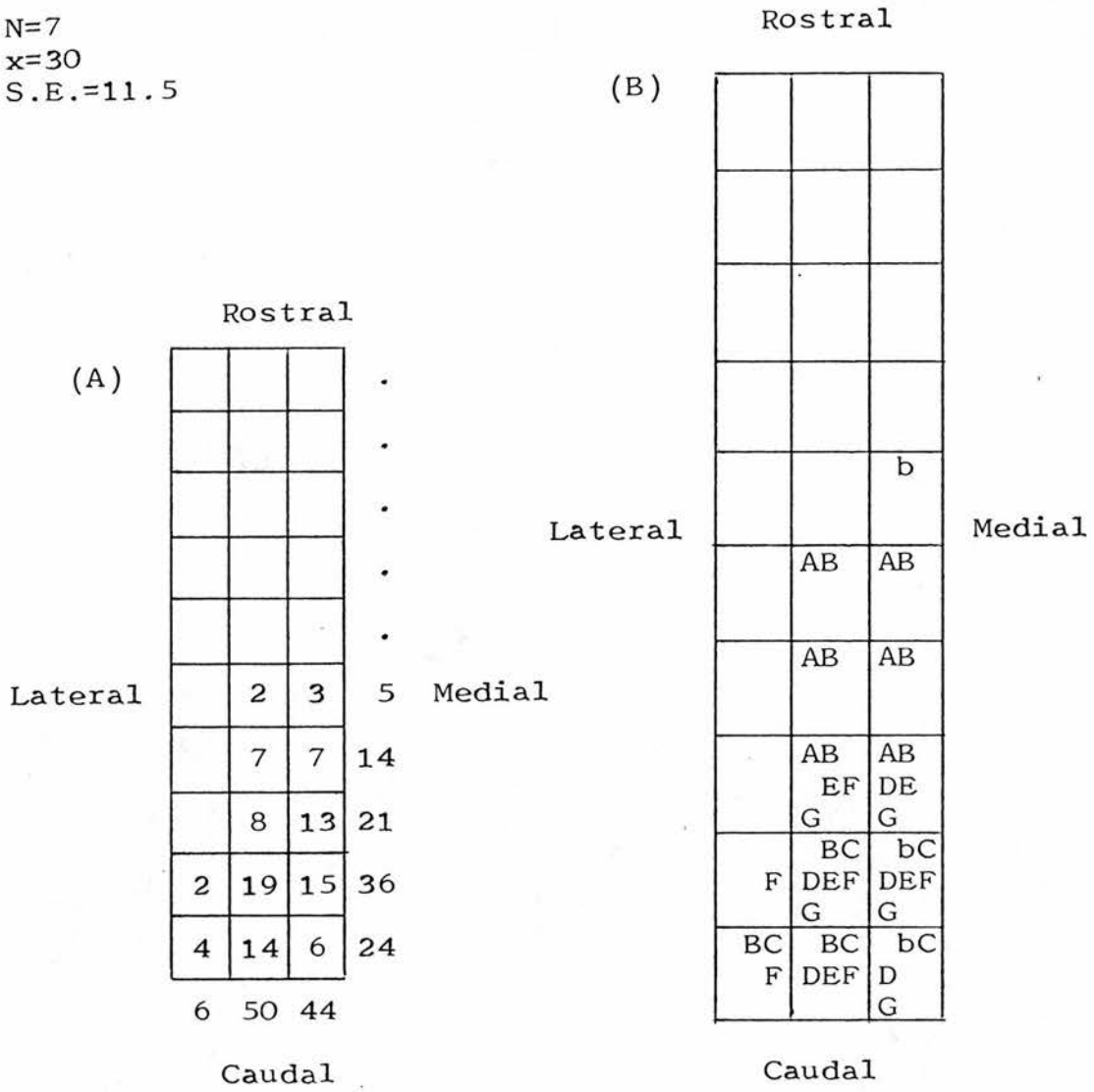


Fig. 6.3

Grids for Plantar injections in the juvenile

(Conventions as for Fig. 6.1).

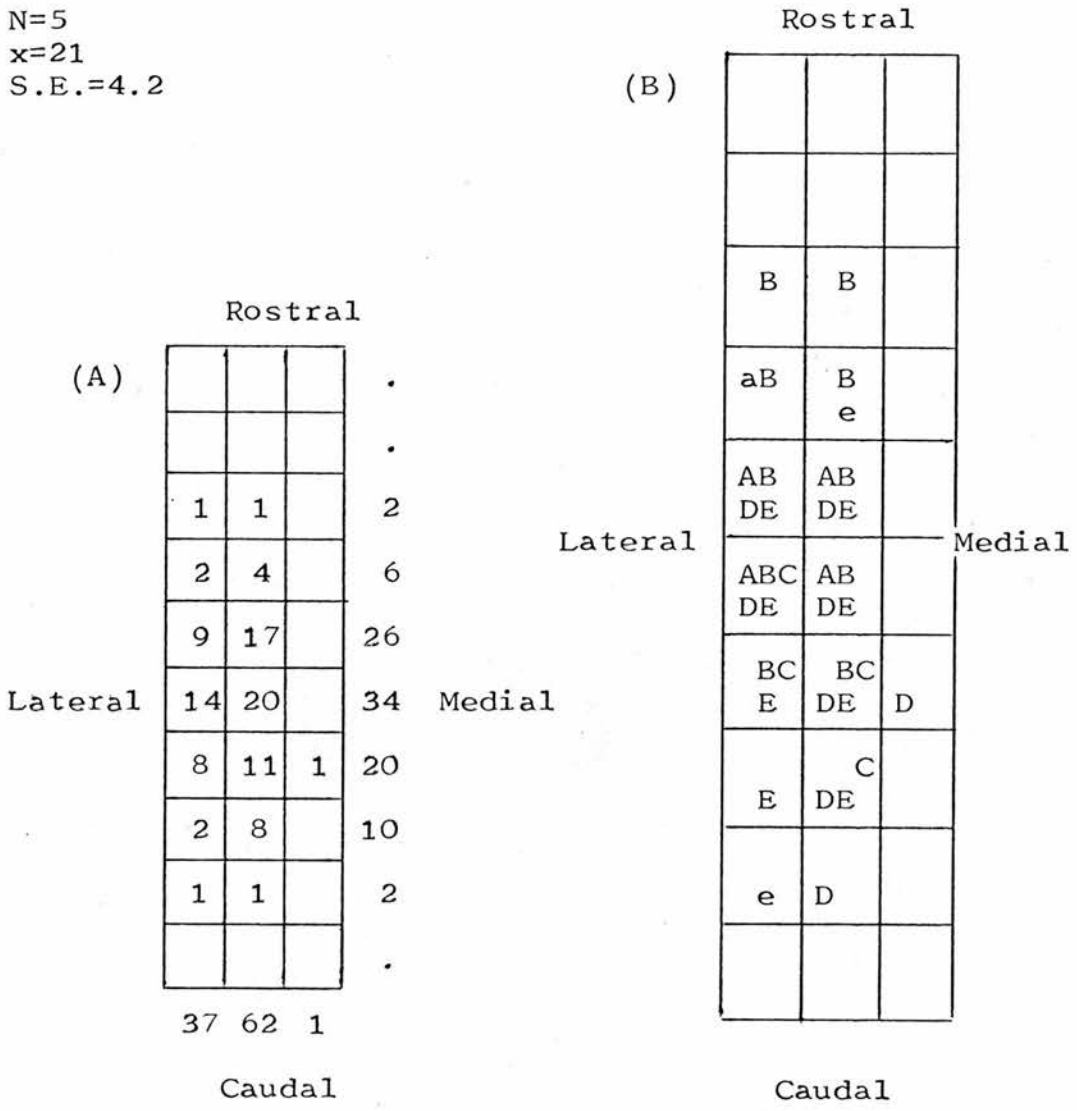


Fig. 6.4

Grids for ALA injections in the juvenile.

(Conventions as for Fig. 6.1).

6.2.3 Innervation pattern to PMT (Fig. 6.2)

Ventral horn cells innervating the PMT fall predominantly into the rostral half of the ventral horn showing a strong medial distribution. Few cells are found in the most caudal three tenths and none most caudally.

6.2.4 Innervation pattern to plantar region (Fig. 6.3)

Ventral horn cells innervating the plantar region are clustered medially in the most caudal extremity of the horn. The more rostral of these cells have a strong medial distribution whereas the most caudal show a more even medio-lateral distribution. The individual patterns (Fig. 6.3 B) fall into two clear groups; a caudal group (C, D, E, F and G) and a more rostro-medial group (A, B). The averaged percentage distribution grids of each group have been assessed separately and compared to the averaged percentage distribution grid for injections into the thenar eminence (short plantar flexors of the first digit) of five Stage 59 animals. There is a clear correspondence of the second group with the patterns for the thenar eminence (Fig. 6.5).

6.2.5 Innervation pattern to ALA (Fig. 6.4)

The ventral horn cells innervating the ALA lie about half way along the horn rather more caudally than rostrally and show a strong lateral distribution. The area from which ventral horn cells send axons to the ALA lies within the area from which ventral horn cells send axons to the ALT.

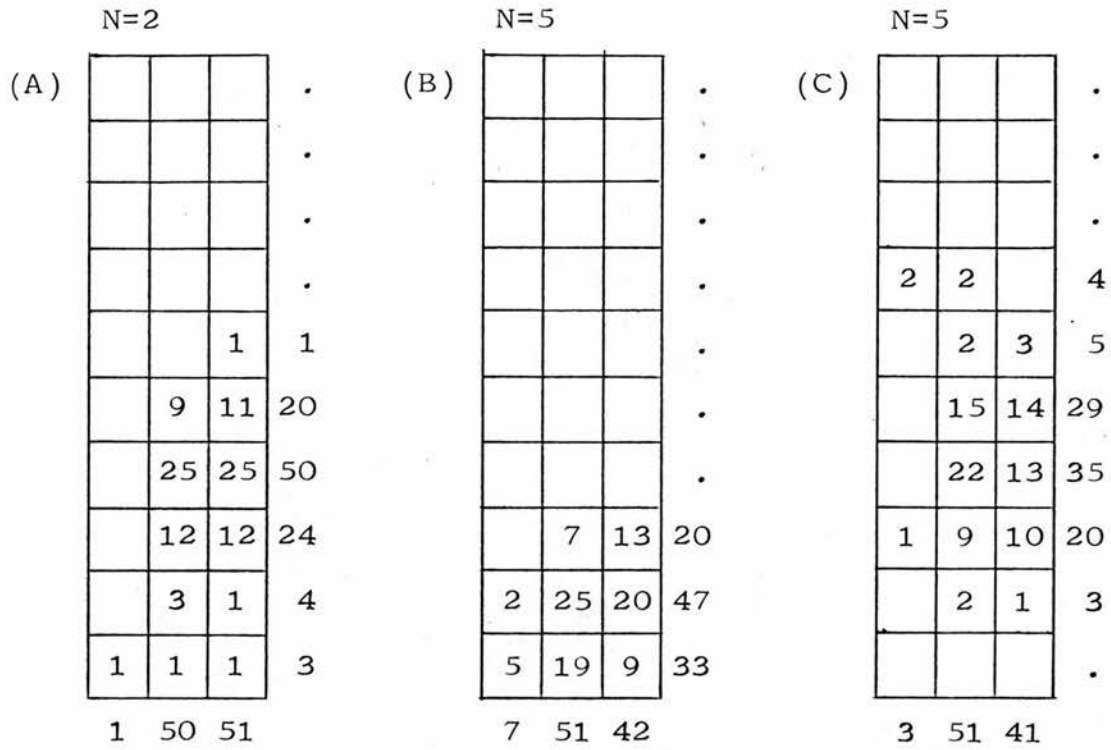


Fig. 6.5

Averaged percentage distribution grids of the two types of labelled cell distribution seen after plantar injections (A and B) and of the distribution seen after injections into the short plantar flexors of the first digit (C). Marked distribution variation for the plantar group (Fig. 6.3) probably results from injection into different intrinsic muscles.

6.3 Discussion and Conclusions

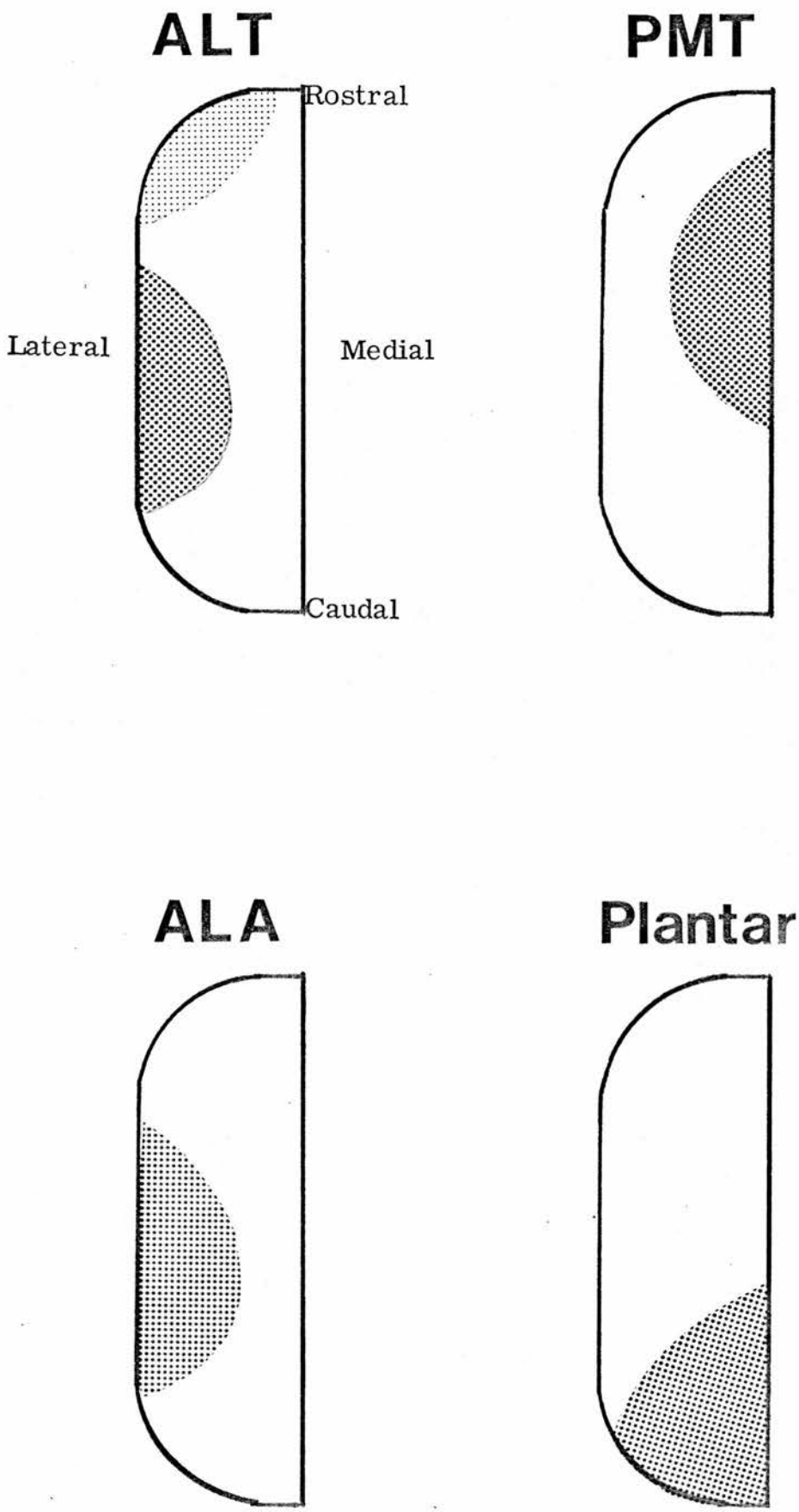
The results for the four regions have been brought together and presented schematically in figure 6.7.

The main considerations in studying these four regions are first, to determine the major qualitative features and differences between them to permit comparison with the features of early patterns. This leads to the second consideration which is that homology must be preserved between the stages in order for the comparison to be valid. This in turn reduces the number of possible areas that can be studied to those which can be clearly separated in the youngest limb buds. For this reason, the results of this chapter are of only limited value for providing an accurate and detailed description of the projection patterns of ventral horn cells to the limb comparable to the studies using chromatolysis described for R. catesbeiana (Cruce, 1974) or the cat (Romanes, 1951). Nevertheless if even the broad characteristics of the few patterns described here can be shown to conform with the more detailed studies cited above then extrapolation of the finer details of those studies to Xenopus might be made with greater confidence.

Before making such a comparison, it is necessary to define in the terms of those previous studies, the areas injected and regions of the ventral horn. Since it is more appropriate to compare this work with that in R. catesbeiana a redefinition of terms using the nomenclature of Cruce, 1974, will be made for the purposes of this discussion.

Fig. 6.7

Schematic diagrams of the ventral horn of Xenopus laevis(Juvenile) to show which regions project to each of the four limb regions studied. Light stippling indicates low density projection. ALT = antero lateral thigh (knee extensors, hip flexors); PMT =postero medial thigh (knee flexors, hip extensors); ALA = antero lateral ankle region (toe dorsiflexors); Plantar = plantar region (toe plantar flexors)


ALT
Rostral
Lateral
Medial
Caudal
PMT
ALA
Plantar

The muscles of the ALT are tensor fascia lata proximally and more distally, gluteus magnus and cruralis. These two muscles form the bulk of this compartment. Iliacus internus and iliacus externus have small extensions into this compartment lying very deep adjacent to the femur. The ALT is therefore virtually identical to the knee extensors in the thigh described by Cruce (1974). The iliacus muscles are described separately as hip flexors and tensor fascia lata receives no mention. In Xenopus this muscle is a hip flexor.

The muscles of the PMT are sartorius, semimembranosis gracilus and adductor magnus. Each of these muscles is treated individually by Cruce except adductor magnus which is not mentioned. All these muscles subserve knee flexion. Sartorius, the gracilus muscles and adductor magnus also are hip extensors.

The muscles of the plantar region are not dealt with as a separate group by Cruce but are included in his ankle and toe extensor group. The plantar muscles of this thesis are the short plantar flexors.

Similarly, the muscles of the ALA are included in Cruce's ankle and toe flexor group. The ALA muscles subserve toe dorsiflexion.

In describing the ventral horn, Cruce uses the terms dorsal and ventral for the most central and the most peripheral ends of the horn as seen in cross section. The equivalent terms used in this thesis are medial and lateral, these terms being derived from the orientation of the ventral

horn in early larvae. To prevent confusion during this discussion the terms will be combined to dorso-medial (DM) and ventro-lateral (VL). This convention has been used by Romanes (1951).

Comparison of the ALT pattern with the knee extensor pattern of Rana shows a strong similarity both in rostro caudal and DM-VL distribution. The more rostral extension of the ALT pattern conforms well with the pattern for the iliacus muscles of Rana and is still in correspondence in the DM-VL axis. The extreme rostral distribution of a minority of cells found in the present study does not have a counterpart in Cruce's work. It is probable that these cells innervate tensor fascia lata which in the adult is at the extreme rostral boundary of the area into which ALT injections are given.

To compare the PMT pattern with Cruce, his patterns for semimembranosis, the gracilus muscles, and sartorius must be examined together. However comparison is made difficult by the absence in his work of a pattern for adductor magnus which forms a major part of the PMT compartment. Nevertheless a good correspondence is seen particularly in the DM-VL axis. According to Cruce, semimembranosis is represented, in part, ventro-laterally, a finding not supported by the present work. Rostro-caudally, the PMT pattern is weighted more rostrally than the combined equivalent in Cruce's work. This difference might be explained by the effect of adductor magnus in weighting the percentage distribution grids of the PMT studies in a rostral direction.

The pattern for the plantar region in this work fits roughly with the pattern shown for the ankle and toe extensors of Cruce. The DM-VL distribution corresponds. However the extreme caudal distribution found for the plantar region does not appear in Cruce's figures. This can probably be explained by the fact that in the category "toe extensors", Cruce includes quite proximal muscles including plantaris. Motoneurones to plantaris in Xenopus were found to be more rostral than motoneurones to the plantar region in a series of Stage 59 animals (Fig. 8.4). The caudal distribution of ventral horn cells supplying only plantar muscles would be masked as a result of Cruce's categorisation. Comparison of the pattern for the plantar region to the patterns for the short toe plantar-flexors of the cat (Romanes, 1951) show a strong similarity along the rostro-caudal axis.

The only source of major disagreement with Cruce is in comparing the ALA pattern with his ankle and toe flexor group. Neither the rostro-caudal nor the DM-VL distributions agree. He finds a very caudal and dorso-medial distribution: the pattern for ALA is much more rostral and definitely ventro-lateral. To obtain the patterns for the ankle and toe flexors, he divided the N.peroneus medial and lateral. These nerves re-unite before supplying the long and short dorsiflexor muscles of the toe (Ecker, 1889). Since these are the muscles contained in the ALA, the explanation for the difference cannot be in the study of non-congruous regions. Interestingly Cruce finds that division of the nerves to peroneus and extensor cruris

brevis produces a quite different pattern although these nerves are branches of the same nerve that gives rise to the medial and lateral peroneus nerves. Division of these nerves leads to changes more rostrally and ventro-laterally which correspond exactly with the changes found for the ALA. Short of postulating interspecies differences in the innervation of this area, no explanation for the difference in the two studies can be given. It should be noted, however, that the distribution described for the Xenopus ALA fits the generalization that embryologically dorsal muscles (which are antero-lateral in Xenopus) are supplied by ventro-lateral neurones whereas the distribution described for Rana toe flexors (the same group) does not. This generalization originally made by Bikeles, 1904, is supported by the present work, the cat study (Romanes, 1951) and largely by the Rana study. Indeed in the cat study the neurones for extensor digitorum brevis and longus lie ventro-laterally and in approximately the same rostro-caudal region as neurones supplying the equivalent ALA muscle of Xenopus. In this respect the findings for the cat and Xenopus agree and, together, differ from the findings for Rana.

The overall conclusion that may be drawn from this study is that the small number of patterns of projection of ventral horn cells to the hind limb in Xenopus which have been examined correspond well to the equivalent patterns for Rana and the cat although certain small differences may exist. The generalization referred to earlier that proximal muscle tends to be supplied by more

rostral neurones than distal muscle is not disproved in this study although since ALT and ALA neurones apparently come from approximately the same rostro-caudal level, it leaves only the comparison between PMT and plantar patterns to support it. A more strongly supported generalization is that referred to earlier (Bikeles, 1904) concerning innervation to embryologically dorsal and ventral muscle, dorsal muscle being supplied by ventro-lateral neurones and ventral by dorso-medial neurones.

CHAPTER 7

PATTERNS OF VENTRAL HORN CELL INNERVATION TO THE HIND LIMB BUDS OF LARVAE AT SERIAL STAGES OF DEVELOPMENT

7.1 Introduction

In this chapter, a direct study is made of patterns of innervation to the four limb regions specified in Chapter 6 at serial stages from the stage of initial motor innervation (Stage 50) to the juvenile. Particular attention is given to the qualitative changes of pattern that occur with development. Further analysis of these changes is made in Chapter 8.

Injections of HRP ranging in volume from about 0.001 µl in the smallest limb buds to 0.1 µl in the largest larval limbs were given into each of the four regions in separate groups of animals at each stage. The manner in which these volumes were measured and their relevance was described in the methods (2.3.2).

In addition, injections were given into the PMT of two other groups of Stage 51 animals. One group had been starved prior to the injection and the other reared and fed for maximum rate of growth (see Methods 2.2.3). All animals were killed and processed 44 hours after the injection. Maps and grids for each group were obtained as described in the Methods (2.10, 2.12).

7.2 Results

7.2.1 Injection sites

Almost all injection sites in young animals (Stage 50-54) were discernible. In older animals, however, injection

sites were often not discernible for the same reasons as occurred in juvenile limbs (6.2.1).

In two (out of ten) Stage 54 ALTs the site was not discernible. However for the reasons given below, these were included in the assessment of the Stage 54 ALT grids (animals B and C, Fig. 7.1 B). In none of the Stage 55 or Stage 59 ALTs was the injection site discernible. In three out of four Stage 55 PMTs and in all Stage 59 PMTs the injection site was not discernible. The single discernible Stage 55 PMT injection site was well localized. In view of the ease of giving injections confined to the intended site in thighs of tadpoles of Stage 54 and more the assumption was made that all injections were correctly sited and all animals in the proximal groups from Stage 54 onwards were included in the assessment of grids. Below Stage 54 all proximal injection sites were discernible and animals were rejected if the injection was not confined to the intended site.

All distal injections were discernible. Only those animals showing proximal extension of HRP were rejected outright. Animals with injections involving both compartments were not used in the assessment of the grids shown for distal regions but were used in the timing observations in Chapter 5.

7.2.2 Innervation patterns to ALT (Fig. 7.1)

The first innervation of this region begins in Stage 50 by a very small number of cells in the medial side of the rostral half of the ventral horn. By Stage 51, a much

Fig. 7.1

Averaged percentage distribution (A) and distribution variation grids (B) of labelled cell distributions seen after injections into ALT at all stages examined from Stage 50 to juvenile. Conventions for N, x and S.E. are the same as defined earlier (Fig. 6.1).

A

Stage 50	Stage 51	Stage 52	Stage 53	Stage 54	Stage 55	Stage 59	Juvenile
N=4	N=11	N=11	N=8	N=10	N=6	N=4	N=5
x=8	x=42	x=34	x=32	x=35	x=44	x=48	x=59
S.E.=0.7	S.E.=10.4	S.E.=10.2	S.E.=8.2	S.E.=7.7	S.E.=7.8	S.E.=17.9	S.E.=17.2

	6		6
	3	8	11
	10	5	15
	19	7	26
	9	5	14
	14	9	23
4			4
			.
			.
			.
4	61	34	

	1	2	3
1	4	3	8
	11	8	19
1	9	11	21
1	7	7	15
1	7	4	12
	3	3	6
	5	3	8
	2	1	3
			.
4	49	42	

1	4		5
3	11	4	18
1	18	5	24
4	11	16	31
1	6	6	13
	4	1	5
1	1	1	3
	1	1	2
			.
			.
11	56	34	

2	13	1	16
9	11	1	21
6	6	1	13
4	10	3	17
1	9	4	14
1	6		7
	4	1	5
1	2		3
	2		2
			.
24	63	11	

12	11	1	24
5	6		11
2	2		4
4	4		8
6	11		17
8	8		16
6	7		13
1	3		4
			.
			.
44	52	1	

8	6		14
5	6	2	13
1	1		2
1	3		4
4	7		11
4	10		14
7	14		21
8	11		19
1	1		2
			.
39	59	2	

3	2		5
3	4	1	8
3	3		6
3	8		11
8	7		15
8	12		20
5	10		15
7	9		16
1	3		4
			.
41	58	1	

	1		1
2			2
3	4		7
3	3		6
5	3		8
15	6		21
19	12		31
13	8		21
2	2		4
			.
62	39	.	

B

	D	
	A	A C
	ABC	C
	AB D	AB
	A C D	A C
	AB D	C D
B		

B f	AB D F g	C F
B F	AB eF G	BC F
B e	ABC DEF GHK	ABC DeF GhK
BC	BC DEF GHK	ABC DEF GHK
B ef G	BC DEF GHK	A C DEF qHK
b qhK	BC DEF GHK	A DEF GHK
ef	B DEF qHK	AbC DEf Gh
	C DEF GHK	DEf GHK
	DEF qhK	A E h
	b F	

d K	C D H	d h
DE	ABC DEF GH	A DE HK
D	AB DEF GHK	AB DE GhK
A DeF H	ABC DeF GH	ABC dEF GHK
D	A C DEF GH	A F GHK
e	ABC E GH	A eF
eF	dE G	F G
	B G	A dE
		G
	G	

ABC F	ABC D F GH	C F
ABC D F	ABC D F G	AB
ABC G	AB EF H	AbC
BC EF H	ABC D F H	ABC E
b F H	AB EF GH	AbC E
B F H	AB DEF GH	Ab
	AB EF G	bC
b EF	Ab EF	b
	E	

A C EF GHK	A C EF GHK	EF Ghk
dEF GHk	AB F GHk	a
a F GH	F GH	
aBC F hK	A C DE hK	
ABC EF GHK	A C DE GHK	
A C DEF GHK	ABC D HK	k
A C D F GHK	A DEF HK	F
A HK	A hK	
E	h	

B DEF	B DEF	d
B DEf	B DEF	deF
b f	B	
Def	AbC dE	
BC DeF	ABC DEF	
AB DEf	ABC DEF	A
ABC DEF	ABC DEF	
AbC D F	ABC DEF	
Ab	B d f	

B	B	
B D	AB D	b D
aB D	BC	
bC D	AbC D	
ABC D	ABC D	
ABC D	ABC D	
AB D	ABC D	
ABC D	ABC D	
ab	ab D	

	A	
a E	e	
DE	A DE	e
AB De	a DE	
ABc Dc	A DE	a
ABC DE	AB DE	
ABC DE	A C DE	
BC DE	A C DE	
BC D	B D	
d		

larger number of cells is innervating this region. These lie in most regions of the ventral horn though only a few are lateral and very few lie in the caudal fifth. The majority tend to be medial and to the rostral side of the midpoint rostrocaudally. This pattern remains virtually the same until Stage 53. Animals at this stage show a range of patterns from the type approximating that seen in Stages 51 and 52 (animal E, Fig. 7.1 B, Stage 53) to a type where labelled cells lie predominantly laterally (F and G, Fig. 7.1 B, Stage 53). The lateral shift is accounted for by a reduction in the numbers of medial labelled cells as well as a marked increase in lateral labelled cells. The averaged percentage distribution grid for Stage 53 (Fig. 7.1 A) shows that the majority of these lateral cells lie rostrally.

By Stage 54, all animals show a lateral distribution of labelled cells. Medial cells no longer label. In addition to the rostral lateral cells, all animals now have a larger group of lateral cells lying more caudally. The result is two groups of labelled cells, one lying at the extreme rostral end and the other centred just caudal to the rostrocaudal midpoint, the two groups merging slightly. This arrangement is maintained in Stage 55 and Stage 59 though the rostral group of cells becomes less prominent. In the juvenile very few cells of rostral group label. Examination of the distribution variation grids shows that an increasing number of animals have no rostral labelled cells in, successively, Stage 55, 59 and juvenile.

Fig. 7.2

Grids for injections into PMT (Conventions as for Fig. 7.1).

A

Stage 50
N=3
x=53
S.E.=11.2

	1		1
			.
1	5		6
4	8	5	17
2	17	6	25
1	8	4	13
1	11	5	17
1	7	1	9
1	4	2	7
	2		2
11	63	23	

Stage 51
N=8
x=147
S.E.=31.6

	1	1	2
1	5	2	8
1	8	2	11
2	9	3	14
2	9	4	15
4	7	3	14
3	8	4	15
1	6	3	10
1	5	3	9
	2	1	3
15	59	26	

Stage 52
N=10
x=113
S.E.=30.3

	2	1	3
1	5	2	8
1	10	3	14
3	10	3	16
4	7	4	15
4	8	4	16
3	5	3	11
3	4	2	9
2	5	1	8
	2	1	3
21	58	24	

Stage 53
N=7
x=162
S.E.=40.2

	3	2	5
1	5	5	11
1	8	7	16
1	8	10	19
1	6	7	14
2	5	5	12
3	3	2	8
2	4	1	7
1	3	1	5
	1	1	2
12	46	41	

Stage 54
N=10
x=184
S.E.=48.0

	3	2	5
	7	8	15
1	12	15	28
	11	11	22
1	6	7	14
1	3	3	7
1	3	2	6
1	1	1	3
			.
			.
5	46	49	

Stage 55
N=4
x=183
S.E.=47.9

	5	3	8
	13	6	19
2	14	11	27
1	13	11	25
1	5	4	10
	3	3	6
	1	2	3
			.
			.
			.
4	54	40	

Stage 59
N=5
x=62
S.E.=11.2

	1	1	2
	5	2	7
	7	12	19
1	11	19	31
3	12	9	24
2	4	3	9
	2	2	4
	1		1
			.
			.
6	43	48	

Juvenile
N=10
x=113
S.E.=28.1

	1		1
	3	3	6
1	7	11	19
1	16	20	37
1	13	10	24
1	6	2	9
1	1	1	3
	1		1
			.
			.
5	48	47	

B

Stage 50

	A	
	c	
B	ABC	
BC	ABC	ABc
Bc	ABC	ABC
C	ABC	BC
A	A C	BC
B	ABC	A C
B	B	A c
	BC	

Stage 51

	C E G	b F h
C e g	BC DEF gh	BC e gH
a C DE G	ABC DEF GH	BC DE gH
a C DE G	ABC DEF GH	BC E GH
AbC DEF Gh	ABC DEF GH	ABC EF GH
AbC DEF GH	ABC DEF GH	ABC De GH
AbC De GH	ABC DEF GH	ABC EF gH
A H	ABC DEF GH	ABC E GH
A h	ABC EF GH	ABc DEF gH
	AbC EF gH	a EF gh

Stage 52

D	aBC eF Ghk	F G k
bC GHk	ABC e GHK	abC G K
c e Hk	ABC DEF GHK	AbC e hK
abC E GHK	ABC DEF GHK	ABC E hK
ABc DE GHK	ABC DE GHK	ABC EF GHK
AB DEF H	ABC DEF GHK	AB DEF hK
B DEF GH	ABC DE GHK	AB DE G K
B DE GH	ABc DE GHK	AB E HK
aB EF G	ABC E GHK	aB E hk
b e	AB E HK	ab E K

Stage 53

c d	A C DE G	A C De G
a de g	ABC DE G	ABC DE G
ab g	ABC DEF G	ABC DEF G
a c F G	ABC DEF G	ABC DEF G
A c de g	ABC DEF G	ABC DEF G
abc eF G	ABC DEF G	ABC DE G
ABC F G	ABC D F G	AbC F G
Abc eF	AbC D F G	A C F g
AbC F g	A C deF	A C d
A g	A C F	A C

Stage 54

A	ABc Def G K	bC D f G k
f k	ABC DEF GHK	ABC DEF GhK
Ab	ABC DEF GHK	ABC DEF GHK
g k	ABC DEF GHK	ABC DEF GHK
d f hk	ABc DEF G K	BC DEF GHK
d k	bc DE GhK	Bc DEf GHK
d HK	DE gHK	dE gHK
e hk	De ghK	de g k
	d	d f g
	d	

Stage 55

b d	ABc D	Ab D
d	ABC D	ABC D
Abc	ABC D	ABC D
bc	ABC D	ABC D
c	BC D	BC D
b	Bc D	BC d
b d	B D	Bc d
b	b	
	b	

Stage 59

	E	B E
e	A c DE	Bc E
e	ABC DE	ABC DE
Bc	ABC DE	ABC DE
B D	ABC De	ABC DE
D	ABc D	A c De
B	A c De	A E
B	B	

Juvenile

f	a EF hk	e h
a f	Ab DEF hk	AB DEF hk
a d h	AB DEF GHK	ABC DEF GHK
ab de Hk	ABC DEF GHK	ABC DEF GHK
a d f hk	ABC DEF GHK	ABC DEF GHK
C d hk	A C DEF HK	AbC dEF hk
ab ef hk	aB ef H	a C ef h
H	C e h	
	f	

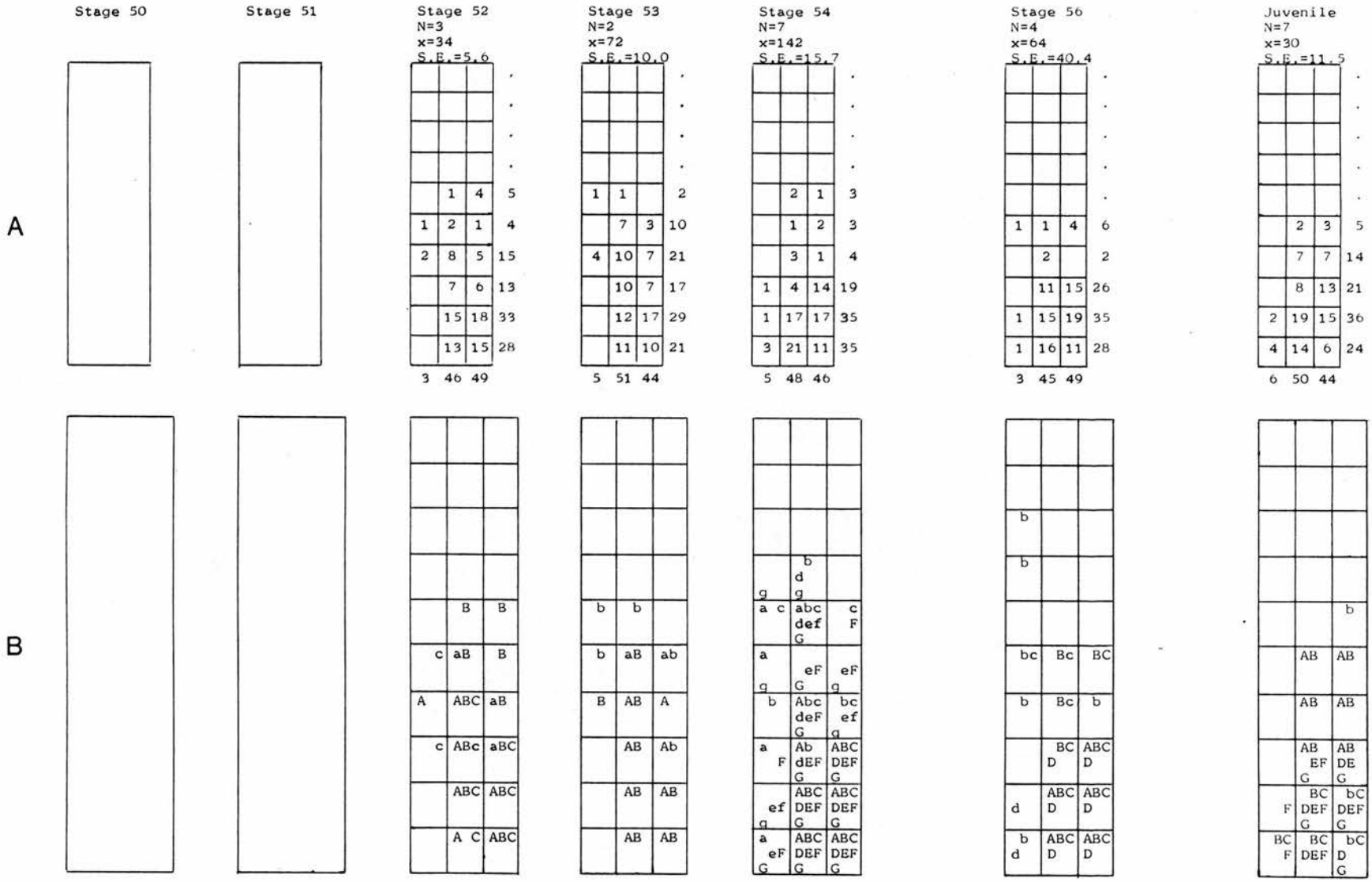
7.2.3 Innervation patterns to the PMT (Fig. 7.2)

The innervation of this region begins in Stage 50 by moderate numbers of cells from most regions of the ventral horn. However only one per cent of cells from the most rostral fifth are labelled. Many more medial cells than lateral cells are labelled. By Stage 51, much larger numbers of cells are labelled. These lie throughout the horn in relatively even distribution. However, the proportion of lateral cells labelled is somewhat smaller. It is to be particularly noted that a good proportion of cells lie at the most caudal extremity of the horn. This pattern remains virtually unchanged until Stage 53 when a fall in the relative numbers of lateral and caudal cells begins. As in the ALT patterns of Stage 53, a range of patterns is seen in Stage 53 PMTs from the type seen in Stage 51 and 52 (e.g. A and C, Fig. 7.2 B, Stage 53) to a type with no caudal and few lateral cells (E, Fig. 7.2 B, Stage 53). By Stage 54, all animals are conforming to the latter type of pattern. In this, there is a strong medial tendency and cells are grouped predominantly in the rostral half of the horn. By Stage 55, only the occasional single cell is seen in the caudal three tenths of the horn and labelled cells are now bunched medially in the rostral half of the horn. This pattern is maintained until the juvenile though the most rostral fifth of the cord shows progressively fewer labelled cells with advancing stage. Animals B and D of Stage 59 show a somewhat aberrent lateral scatter of labelled cells in addition to the normal distribution for late larval and juvenile

Fig. 7.3

Grids for injections into Plantar

(Conventions as for Fig. 7.1).


Stage 50
Stage 51
Stage 52
N=3
x=34
S.E.=5.6
Stage 53
N=2
x=72
S.E.=10.0
Stage 54
N=7
x=142
S.E.=15.7
Stage 56
N=4
x=64
S.E.=40.4
Juvenile
N=7
x=30
S.E.=11.5
A
B
3 46 49
5 51 44
5 48 46
3 45 49
6 50 44

stages. It is possible that the injections involved the knee-extensor muscles to a minor degree. Unfortunately this could not be ascertained by examination of the injection site for the reasons given earlier (7.2.1).

7.2.4 Injections in the foot

The foot paddle does not become visible as a distinct entity until Stage 52. However injections were given as distally as possible into Stage 50 and 51 limb buds. These injections gave uniformly negative results (5.3). It was found to be very difficult to give injections into either the plantar or dorsal aspect alone of the paddle at Stages 52 and 53. Most animals were rejected at the time of injection since HRP was clearly seen to enter both compartments. Of the 28 Stage 52 animals not rejected at the time of injection, 18 showed no uptake and, on histological examination, five of those with uptake showed involvement by the injection of both compartments. This left only three animals with pure plantar and two with pure ALA injections in which any cells were labelled. Similar problems arose in obtaining sufficient animals of Stage 53 with pure plantar injections.

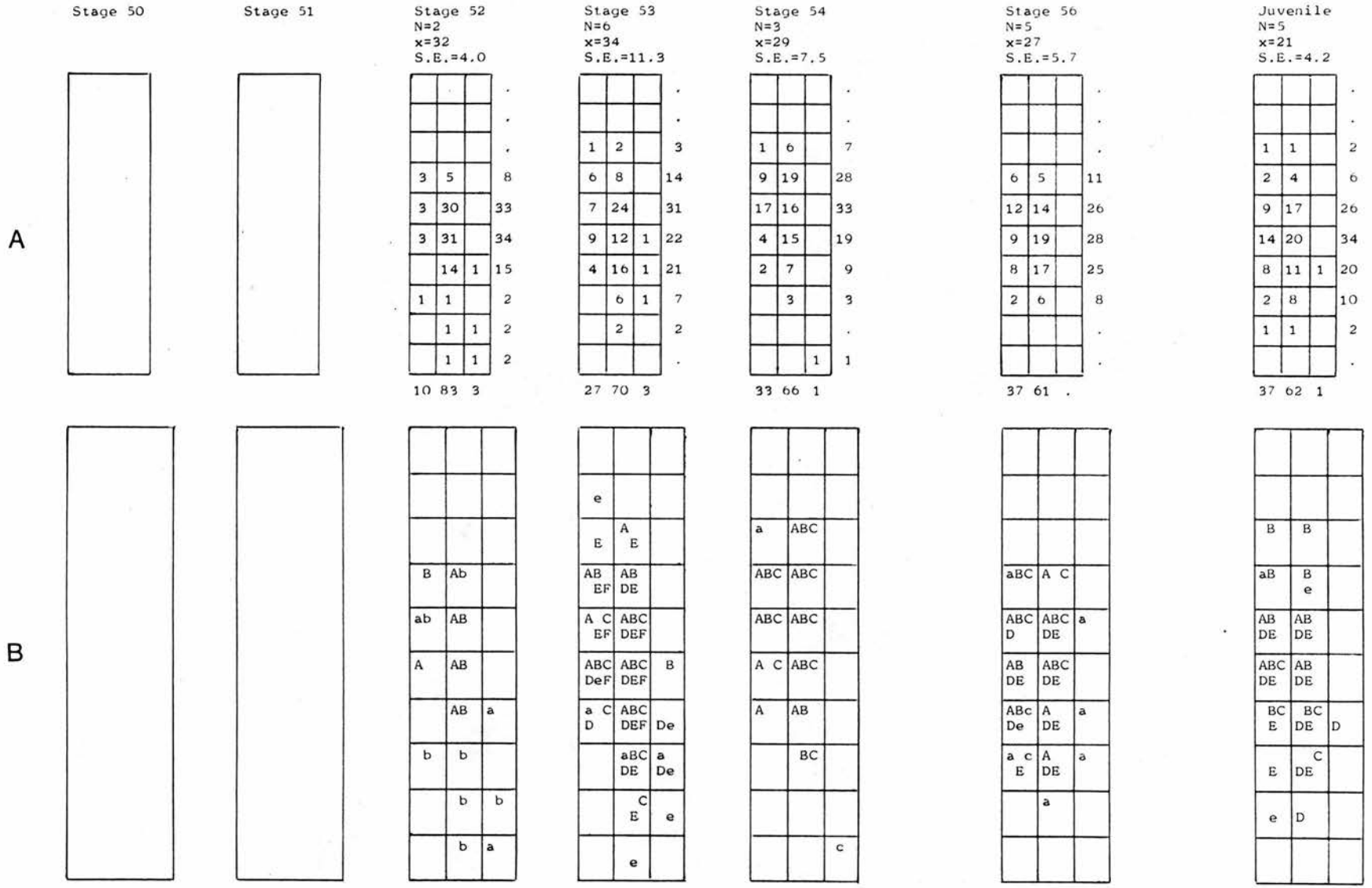
7.2.5 Innervation pattern to the plantar region (Fig. 7.3)

At all stages from Stage 52 to juvenile, the same region of the horn innervates the plantar aspect of the foot. The labelled cells lie predominantly in the caudal three or four tenths and are especially dense in the most caudal fifth. Very few lateral cells are labelled at

Fig. 7.4

Grids for injections into ALA

(Conventions as for Fig. 7.1).


Stage 50
Stage 51
Stage 52
N=2
x=32
S.E.=4.0
Stage 53
N=6
x=34
S.E.=11.3
Stage 54
N=3
x=29
S.E.=7.5
Stage 56
N=5
x=27
S.E.=5.7
Juvenile
N=5
x=21
S.E.=4.2
A
B
10 83 3
27 70 3
33 66 1
37 61 .
37 62 1

Stages 52 and 53 the large majority tending to be medial but from Stage 54 onwards, a moderate proportion of lateral cells is labelled in addition to the medial cells.

7.2.6 Innervation patterns to the ALA (Fig. 7.4)

As for the plantar patterns, the patterns of cells innervating the ALA remains virtually the same from Stage 52 to juvenile. Only one change with development is seen. At Stage 52, few lateral cells are labelled. Most labelled cells lie in the middle of the horn as seen in transverse section. During Stage 53 and 54 the proportion of lateral cells increases to the adult level and remains unchanged for the rest of larval life. It should be noted that at no time do significant numbers of medial cells innervate the ALA.

7.2.7 The numbers of cells innervating each region (Table 7.1)

At all early stages, about four times as many cells become labelled after PMT injections as after ALT injections. It is likely that these figures represent real differences in innervation density to the two regions in terms of ventral horn cells per unit volume of limb tissue, though this conclusion must be regarded with some caution (see 2.13).

To begin with, the numbers of labelled cells after plantar and ALA injections are not significantly different though plantar injections give increasingly higher labelled cell numbers up to Stage 54 and thereafter, lower numbers.

A

Stage	ALT		PMT		$p[\bar{x}(ALT)=\bar{x}(PMT)]$ Students' t' test	Significant difference in means ? (5% level)
	$\bar{x}$	S.E.	$\bar{x}$	S.E.		
50	8	1.0	53	11	< 0.005	Yes
51	42	10	147	32	< 0.005	Yes
52	35	10	113	30	< 0.02	Yes
53	31	8	162	40	< 0.005	Yes
54	35	8	184	48	< 0.02	Yes
55	43	8	183	48	< 0.01	Yes
59	48	18	62	11	< 0.6	No
J	59	17	113	28	< 0.3	No

B

Stage	Plantar		ALA		$p[\bar{x}(Plantar) = \bar{x}(ALA)]$ Students 't' test	Significant difference in means ? (5% level)
	$\bar{x}$	S.E.	$\bar{x}$	S.E.		
50	-	-	-	-	-	-
51	-	-	-	-	-	-
52	34	6	32	4	< 0.9	No
53	72	10	38	11	< 0.2	No
54	142	16	29	8	< 0.005	Yes
56	65	40	27	6	< 0.4	No
J	31	12	21	4	< 0.7	No

Table 7.1

A: Mean labelled cell numbers ($\bar{x}$) and Standard errors (S.E.) for ALT and PMT injections with comparison of the means, for each stage examined.

B: Similar tabulation for plantar and ALA injections.

J = juvenile.

However, only at Stage 54 is there a significant difference of labelled cell numbers for the two regions suggestive of a difference in innervation density.

7.2.8 Comparison of Stage 51 PMT innervation patterns in starved and fast growing animals (Fig. 7.5)

Both groups show differences in pattern from the Stage 51 PMT pattern of the main series (7.2.3). The essential feature of the starved group pattern is the even medio-lateral spread of labelled cells, giving as many lateral labelled cells as medial. The rostro-caudal distribution is the same as in the main series. The fast grown group pattern shows an almost total absence of lateral labelled cells and again the rostro-caudal distribution is almost the same as in the main series. The average number of labelled cells in the fast grown group was 190 and in the starved group, 99. However great variation in both groups makes the difference not statistically significant ($p = 0.10$ students 't' test). The average cell number for the main series group was 147 which is also not significantly different from either starved or fast grown groups.

7.3 Discussion and Conclusions

7.3.1

The essential features and changes in the pattern of innervation to the four regions at serial stages are presented schematically in Fig. 7.6. The important features are:

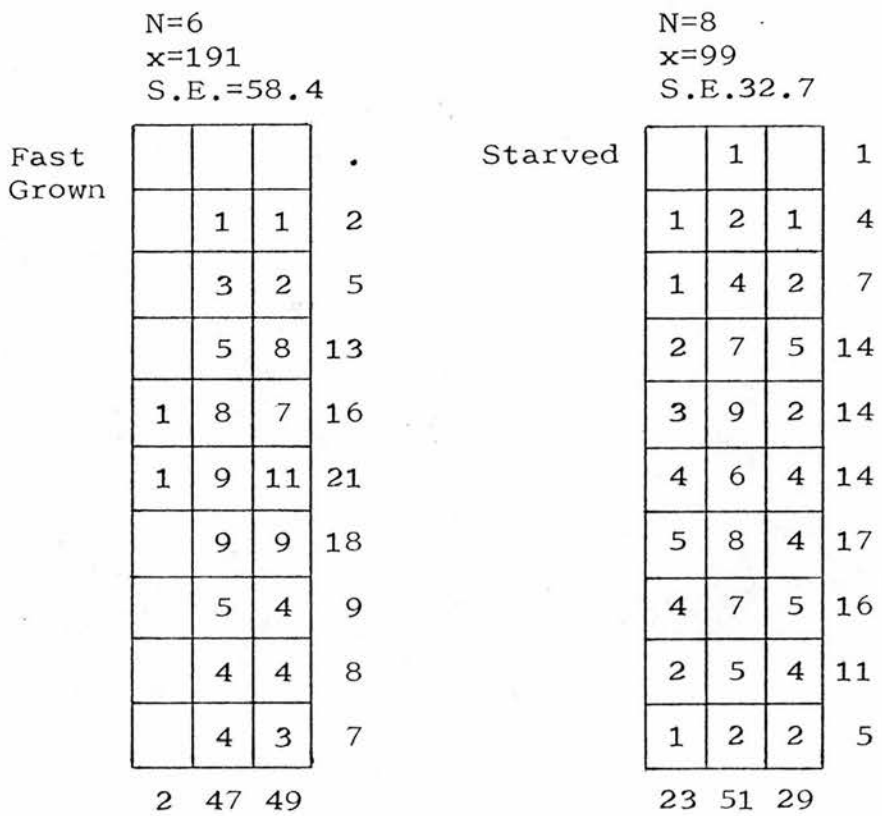


Fig. 7.5

Averaged percentage distribution grids for PMT injections in fast grown and starved groups of Stage 51 animals. Note difference in medio-lateral distribution.

1. In early stages (Stages 50-52) caudal ventral horn cells innervate the PMT and in later stages (Stage 54 to juvenile) they do not.

2. In early stages medial ventral horn cells innervate the ALT and in later stages they do not.

3. While there are these major differences between early and late patterns of innervation to the thigh the earliest patterns to the distal regions are similar to the late stages. However, the earliest innervation to the distal regions does not occur until Stage 52.

4. In early stages the proportion of lateral motoneurones innervating all four regions is low.

5. The proportion of lateral ventral horn cells innervating the PMT falls even further in later stages while it rises in the other three regions.

6. All these changes begin in Stage 53.

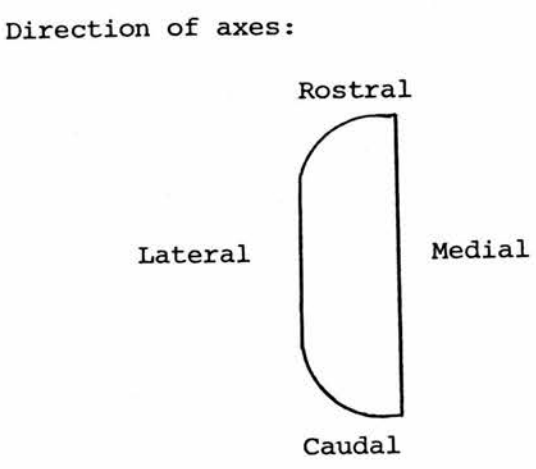
7. In the late larval stages, the proportion of the most rostral ventral horn cells innervating the two thigh regions appears to fall.

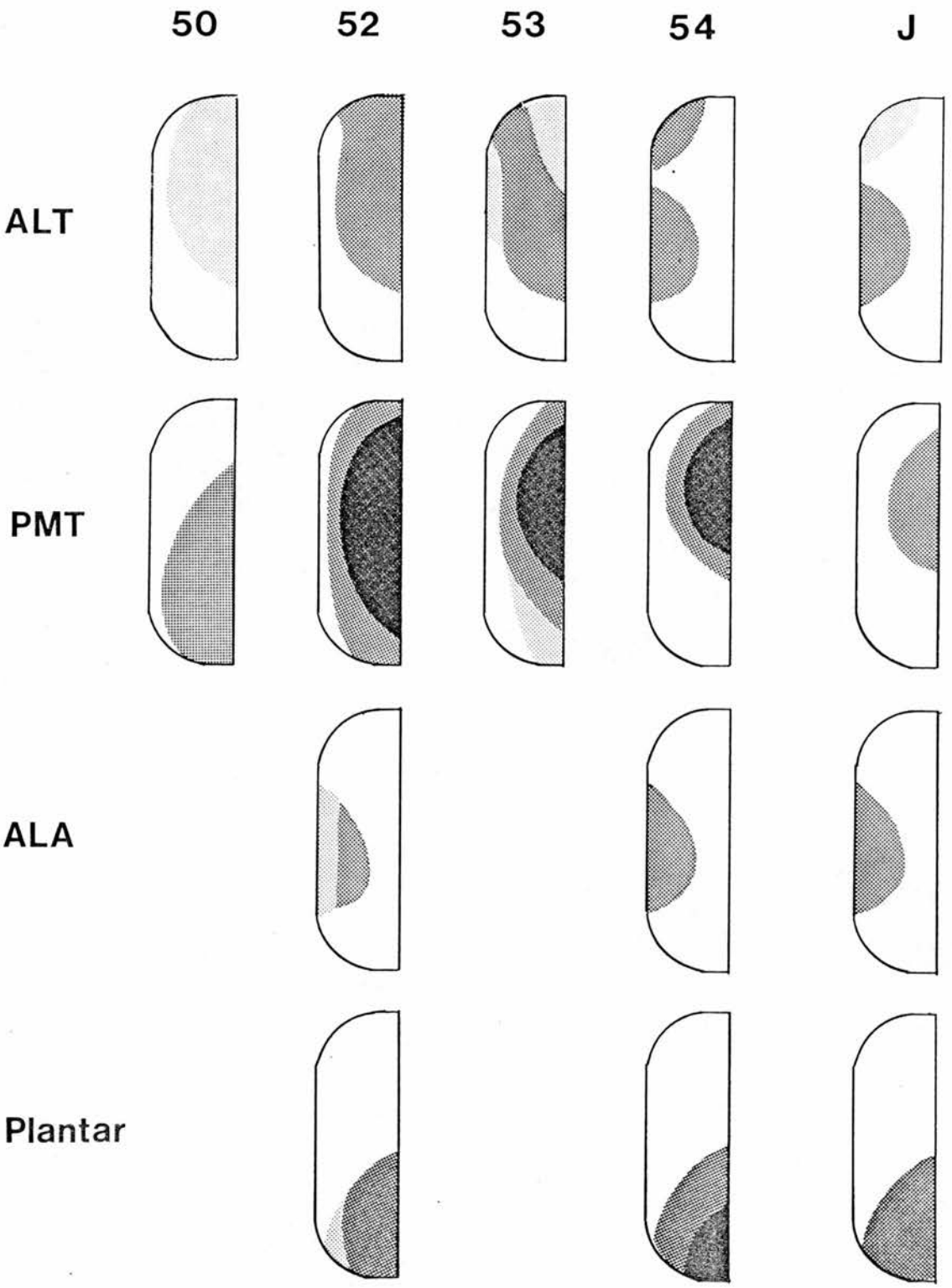
7.3.2 Innervation patterns

In considering many of these features, it is necessary to recall some of the points made in Chapter 1 and in particular the temporal relationships of developmental events in the cord and the limb. Both regions of the thigh under investigation are determined simultaneously by early Stage 50 (Tschumi, 1957). However the ventral horn cells supplying each region in the adult are generated at widely different times. The ventral horn cells supplying the PMT, being

Fig. 7.6

Schematic diagrams of the ventral horn of Xenopus laevis to show the essential features of the patterns of projection to the four limb regions studied, at successive stages (Nieuwkoop and Faber 1967). Patterns determined by the retrograde axonal transport of horseradish peroxidase from the limb to the ventral horn cell bodies. Only stages in which a change of pattern has occurred from that seen at an earlier stage are shown. Density of stippling represents projection density. J = juvenile; ALT = antero lateral thigh; PMT = postero medial thigh; ALA = antero lateral foot and ankle region; Plantar = postero medial foot and ankle region. (see Fig. 1.1)




50
52
53
54
J
ALT
PMT
ALA
Plantar

medial in the adult, are laid down from Stage 50 since the medial ventral horn cells are the first to be laid down (Prestige, 1973). However, the ventral horn cells which supply the ALT, being lateral in the adult, are not laid down until later in ventral horn development. Many if not all of these may not be laid down until Stage 52 to Stage 54.

The important conclusion arising from the results of injections into the ALT at early stages is that this region is nevertheless innervated. Not only are many of the cells innervating the region medially situated, but it can also be argued from what was said in the preceding paragraph that all ventral horn cells at these stages are effectively medial with respect to their eventual adult position. This means that at early stages, particularly Stages 50 and 51 the ALT is innervated entirely by a population of cells not appropriate to the adult pattern and none of which will be innervating it after Stage 53.

The two regions of the foot and ankle under study are not determined until Stage 52 (Tschumi, 1957). However, as early as Stage 50, some ventral horn cells are laid down in the most caudal medial part of the ventral horn, the region that in the adult will be innervating the plantar region of the foot. All ventral horn cells grow their axon immediately upon arriving in the ventral horn (Prestige and Wilson, 1972, 1974) and they reach the limb in a very short time (Lamb, 1974; Prestige and Wilson - personal communication). The most caudal cells laid down in Stages 50 and 51 must therefore innervate the limb despite

the absence of the foot and ankle. Since no motor axons innervate the distal part of the limb until Stage 52 (Chapter 5), their endings must lie proximally. The results of this chapter show that they lie mainly in the PMT. Few innervate the ALT.

In contrast to the situation in the thigh at the time that the distal regions begin to be innervated (Stage 52), it is likely that there is a population of ventral horn cells available appropriate to each region. Nor are there any populations of ventral horn cells now without a limb region which it is appropriate for them to innervate. It is possible that these explain why innervation to distal regions is "correct" from the earliest innervation. There are no inappropriate ventral horn cells to innervate the plantar region as a result of being unable to go to their appropriate region (as occurs in the PMT). However, it is more difficult to explain why no medial ventral horn cells should inappropriately innervate the ALA as occurs with the ALT. It could be postulated that the lateral ventral horn cells innervating the ALA have the power to prevent innervation by medial ventral horn cells. Alternatively, the different manner of innervation between proximal and distal regions might be the result of other factors. This will be discussed further in Chapter 9.

The question of what happens to the ventral horn cells innervating the PMT and ALT which are inappropriate to the adult pattern is dealt with in Chapter 8.

7.3.3 Are the labelled cell patterns representative of the manner of initial fibre ingrowth?

The early labelled cell patterns are ordered. Is initial fibre ingrowth therefore similarly ordered? It is necessary to ask whether there is a phase of innervation by each fibre occurring before it becomes detectable by the present technique. This phase might differ from that represented by the labelled cell patterns. Fibre endings might move at random to many limb regions (relocation), or branch to many regions with degeneration of all but one branch (branch degeneration) before becoming detectable. Fibres in such a phase of innervation might be undetected either because of a failure of the cell bodies to become labelled or because they belong to a minority too small to show in the labelled cell patterns. Failure to label may result from the retrograde transport system beginning to operate only after the completion of the postulated earlier innervation phase, or because fibre endings are in contact with the injection site too briefly to take up adequate amounts of HRP. An undetectable minority of labelled cells would result if HRP is available for uptake for only a very short time and all fibres are in the postulated earlier innervation phase for only a very short time.

These points will be dealt with separately.

1. Delayed or non-operation of the retrograde transport system: Arguments have been presented already (3.8) to support the view that even growing endings are capable of taking up HRP. However it must be emphasised that the in vivo evidence for this is not good, nor is there any

evidence that there are not some endings which are silent as far as HRP uptake is concerned. Although it seems unlikely that a situation of differential HRP uptake should exist according to the location of the endings, the possibility cannot be excluded and must be borne in mind. However, it can be said that fibres need not reach a site appropriate to the adult pattern before commencing detectable HRP uptake. This is seen from the early thigh labelled cell patterns.

2. HRP not taken up in detectable amounts due to too short a contact with the injection site: This is unlikely if it is assumed that HRP is available for uptake for as little as one hour as is the case in rat intercostal muscle (Zacks and Saito, 1969). It is certain that some endings will be in contact with even the smallest HRP boluses for this time since their maximum rate of growth is only half the diameter of the smallest boluses per hour (45 μm/hour - Speidel, 1941, 1942) and this does not allow for an apparent sampling time during which they are stationary (Chalmley et al., 1973).

3. Too small a proportion of endings undergoing relocation or branch degeneration during a short pulse of HRP availability to be detectable despite adequate HRP uptake: Suggestive evidence that this does not occur comes from the results of experiments to be described in Chapter 8. In these, it is shown that while large numbers of cells innervating the PMT at Stage 51 survive until Stage 56, none innervating the ALT at Stage 51 do. If some ventral horn cells innervating the PMT had previously innervated the ALT

for a brief time, then after injections into the ALT at Stage 51, a detectable number of ventral horn cells should have become labelled before moving to the PMT. Of these, some might be expected to have survived until Stage 56 since many ventral horns cells innervating the PMT at Stage 51 do survive this period. Yet no labelled cells did survive.

In conclusion, it is felt to be unlikely that an early innervation phase differing from that represented by the earliest HRP detectable patterns has been missed.

It can be argued that even if an early phase of relocation and/or branch degeneration does occur, it is of little relevance to the present study. One object of determining the early HRP labelled cell patterns was to see if fibres initially innervate the limb in an ordered or an unordered fashion. Individual neurites growing in vivo (Speidel, 1941, 1942) or in vitro (Harrison, 1910; Chalmley et al., 1973) grow by extending numerous filaments from the growth cone. One or more of these may thicken and elongate to form the further paths of the growing ending though usually all but one eventually retract. Single growing endings may also retract and begin growing in a new direction. Each of these processes could be described as branching plus branch degeneration or relocation of endings respectively. The purpose of these processes is presumably to enable the growing ending to choose a suitable pathway (Sperry, 1963). Thus ordered ingrowth may necessarily be preceded by a brief period of what could be termed relocation or branch degeneration. However, the

interest lies in the manner in which fibres become committed to given regions. During ingrowth, decisions must be made about which filaments to grow along, which branches to retain or whether to keep moving. The final site of innervation is the culmination of these decisions. In this thesis, fibre ingrowth will be defined as ordered or unordered only in relation to the location of the endings after these decisions have been taken. It is likely that the HRP labelled cell patterns accurately reflect this phase of innervation.

7.3.4 Labelling of the lateral ventral horn cells

The proportion of lateral ventral horn cells innervating all four regions in early stages is low. Newly generated neuroblasts accrete to the lateral side of the ventral horn (Prestige, 1973). If a certain amount of time must elapse after joining the ventral horn before new neuroblasts can grow an axon into the limb and hence take up HRP then there will always be a band of neuroblasts laterally which are unlabelled so long as new neuroblasts continue to be laid down. The thickness of the band will depend on the rate of accretion of new cells and the length of time required for the axon to reach the limb. This idea can be tested by changing the production rate of neuroblasts and observing the thickness of the unlabelled lateral band. Production of neuroblasts can be completely halted by total starvation (Prestige - personal communication). The PMT maps obtained from starved Stage 51 animals (Fig. 7.5) show abundant numbers of lateral labelled cells equal in

number to medial labelled cells while maps obtained from fast grown animals (Fig. 7.5) with a high production rate show very few lateral labelled cells. This conforms with the predictions of the model. These results also provide confirmatory evidence that neuroblasts are laid down in a medio-lateral order, since this assumption was made in proposing the model.

The main series Stage 51 and 52 patterns show a moderately high proportion of laterally labelled cells. At the time of these experiments many animals in these groups were noted for being slow-growing. The results of the starvation experiment confirm that slow growth was the likely reason for the fairly high proportion of lateral labelled cells in these animals.

Arising from these observations is the point that there is a fundamental difference between the cells labelled as lateral up to about Stage 52 and those labelled as lateral thereafter. The former will become more medial as new cells are laid down and cannot be thought of as truly lateral in the adult sense. This is an important distinction to be kept in mind when considering mechanisms of innervation (Chapter 9).

7.3.5 Starvation and axon growth

The results of the starvation group show that starvation does not cause the cessation of axonal growth at least in the time and over the distance required for axons to grow from the ventral horn to the limb at Stage 51. This seems to be in apparent contradistinction to sensory axons growing

in tadpole tail which cease elongation and begin to retract with starvation (Speidel, 1942). However, the periods of time over which the two types of fibres were observed may have differed. Ventral horn cell axons may also cease growing if given further to grow or more time before reaching the limb.

7.3.6 Rostro-caudal time gradient in pattern development

The grids of the ALT patterns of Stages 53 and 54 show that the lateral predominance of labelled cells begins rostrally and takes about a stage to be complete along the rostro-caudal axis. This conforms with the rostro-caudal time gradient of development in the ventral horn.

7.3.7 Homology of injection sites

In these experiments the assumption was made that the regions described as ALT and PMT in Stages 50 and 51 before the plane of the limb bud is visible, are homologous with the ALT and PMT of later stages. However such an assumption can only be true if there is no change of the position of limb regions relative to the body axes which were used for defining the regions. For example, it is possible that rotation of the limb about its proximo-distal axis might occur. A satisfactory proof of homology of the thigh regions over the early stages up to Stage 56 is derived from the results of Chapter 8 and will be presented there.

However, the present results suggest that complete homology is not maintained at later stages. When the adult patterns for ALT and PMT first develop at about Stage 54, a

large number of rostral cells innervate both regions. With development, progressively fewer rostral cells innervate these regions until in the juvenile, none of the most rostral cells innervate PMT and few innervate ALT. The most likely explanation of this is that the most rostral cells innervate muscles of the hip region. At Stages 54 and 55, the hip region is usually involved in the injection site but growth of the limb and pelvis puts the hip region progressively beyond the boundaries of ALT and PMT injections. However, this assumption has not been tested.

7.3.8 Further evidence of the non-importance of diffusion

The results of the distal injections provide further evidence that diffusion of HRP does not take place over distances large enough to influence the labelled cell patterns presented in this thesis. Injections into the plantar region and ALA result in different and non-overlapping patterns of labelled cell distribution even as early as Stage 52. At Stage 52 the distance between these two regions is less than 100 μm, thus diffusion can take place only over a distance less than this.

7.3.9 Conclusion

The results of this chapter have shown that the initial patterns of innervation as shown by HRP labelling are ordered. Each region tested is innervated in a distinctive way. However, the early thigh patterns of innervation are very different from the juvenile thigh patterns. The early patterns have been related to disparities in the timing of development

of limb and ventral horn regions though other factors may also play a role (Chapter 9).

The rearrangement of the innervation patterns from the early to the late types has been shown to occur in Stage 53. This is dealt with further in the following chapter (Chapter 8).

CHAPTER 8

THE FATE OF VENTRAL HORN CELLS AND THEIR AXONS INNERVATING MUSCLE REGIONS INAPPROPRIATE TO THE ADULT PATTERNS

8.1 Introduction

As shown in Chapter 7, two major changes of pattern take place in Stage 53. First, caudal ventral horn cells innervating the PMT cease to do so and secondly, medial ventral horn cells innervating the ALT cease to do so. There are three possible ways in which this could happen. They are based on the theoretical mechanisms discussed in 1.4 and two of them were discussed in a different context in Chapter 7 (7.3.3).

1. The axon endings move away from the region which they have been innervating and innervate regions appropriate to the adult patterns (relocation).

2. The axons initially branch widely to many areas and the change is caused by the retraction or degeneration of branches inappropriate to the adult pattern leaving only the appropriate branches (branch degeneration).

3. The moto-neurones die (cell death).

In addition a fourth theoretical possibility exists that the ventral horn cells move into the region of the ventral horn appropriate to the muscle region to which they are connected. The converse is that the muscle cells move. These possibilities can be excluded immediately. In an auto-radiographic study of ventral horn development (Prestige, 1973), it was shown that the medio-lateral order of laying down of cells was not disturbed by subsequent migration of cells either medially

or laterally from their initial position. Any massive migration such as would be required to explain the medial to lateral shift of pattern for the ALT would be easily detectable by this method. In order for muscle cell migration to fit the patterns, large numbers of muscle cells initially residing proximally would have to migrate distally to form the foot. Absence of any distal migration of muscle cells in limb formation has been shown by Tschumi (1957) in normal Xenopus hind limb by Cairns (1965) using mouse-chick interspecies mesenchymal grafts and by Amprino (1965) using mesenchymal grafts between chick wing and hind limb buds.

The experiments described in this chapter were designed to distinguish between cell death on the one hand and relocation or branch degeneration on the other as the means by which the patterns change. The rationale of the approach is shown in Figures 8.1 and 8.2. Injections are given into the PMT or the ALT respectively in the two experiments at Stage 51 when the early patterns are well established but are still some time away from the changeover point when the adult patterns emerge (Stage 53). After the changeover point, that is from Stage 54 onwards, the animals are killed and the distributions of labelled cells mapped in the usual manner. If all cells which have become labelled prior to the changeover point survive after the changeover point then the pattern which will be found on examination will be the same as if it had been examined at Stage 51. If however, all cells inappropriate to the adult pattern which have become labelled in Stage 51 die at

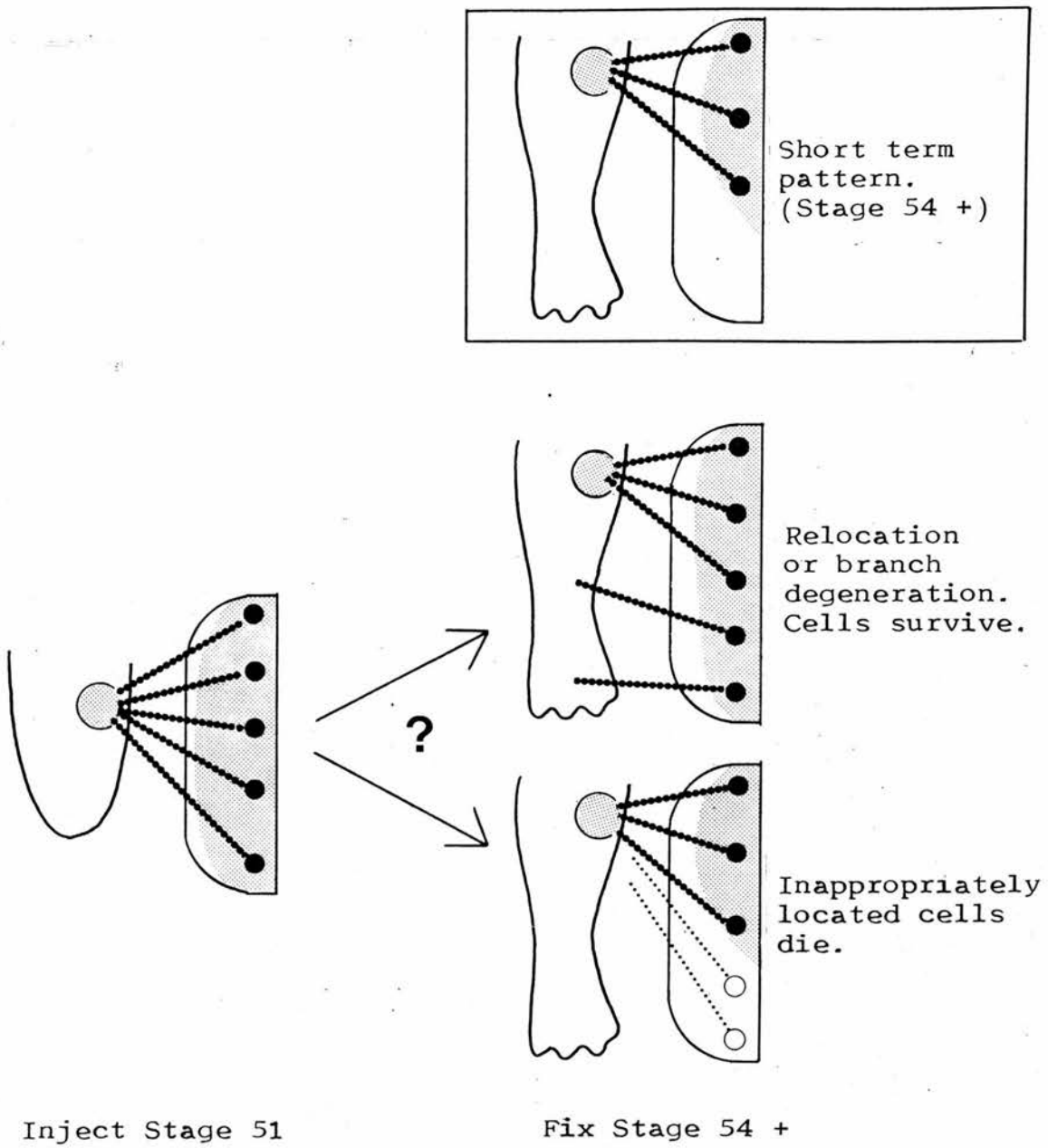


Fig. 8.1

The rationale of the delay PMT experiment: If caudal ventral horn cells which became labelled by a PMT injection at Stage 51 relocate their endings and survive until Stage 54 or more, then the rostro-caudal distribution of labelled cells will be the same whether examined at Stage 51 or 54 or more. If they die however, the rostro-caudal distribution when examined at Stage 54 or more will be the same as if the injection had been given at Stage 54 or more. Inset shows short term projection pattern at Stage 54 or more.

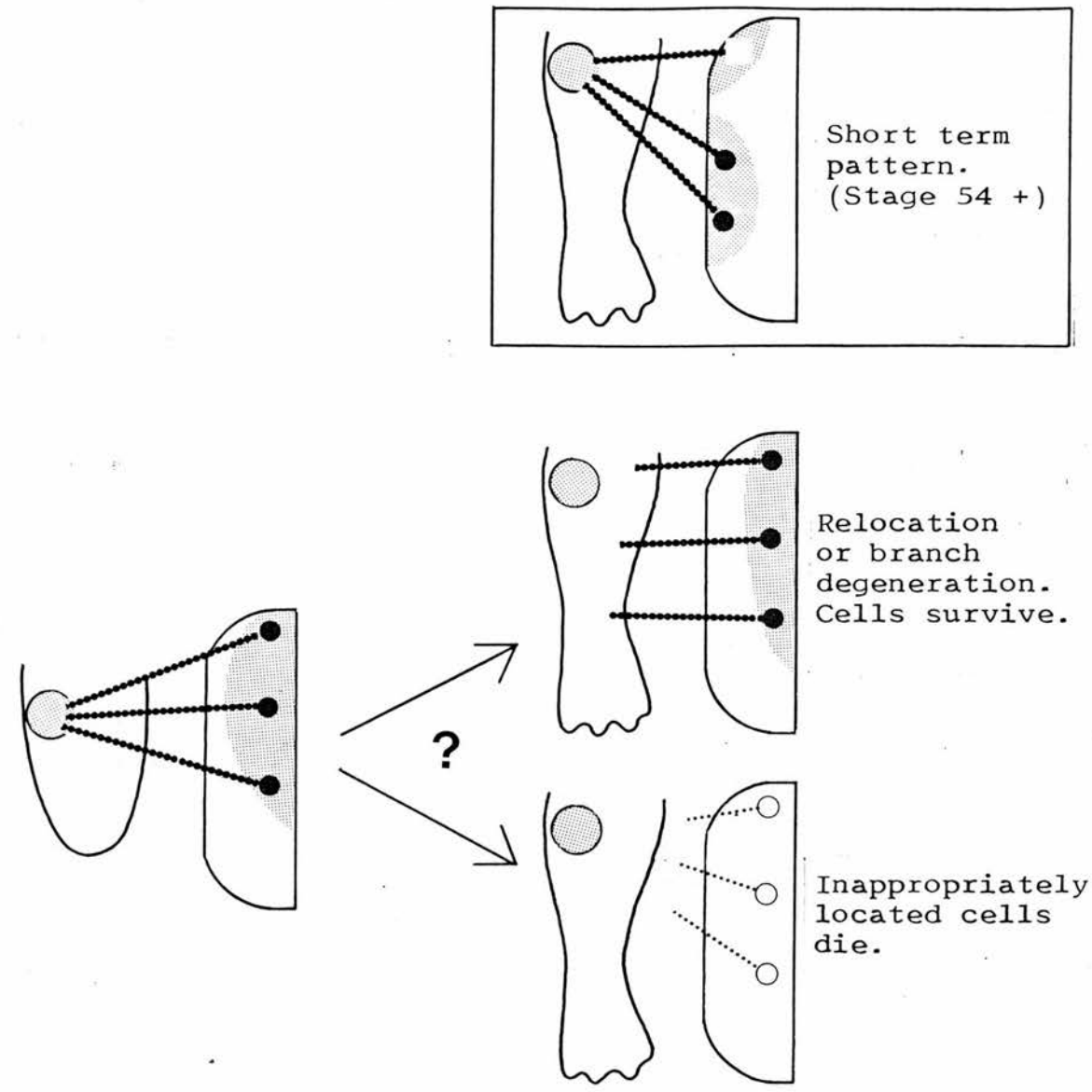


Fig. 8.2

The rationale of the delay ALT experiment:
If the early (Stage 50 and 51) ventral horn cells which become labelled by an ALT injection at Stage 51 relocate their endings to postero-medial muscle and survive then the distribution of labelled cells found will be the same whether examined at Stage 51 or Stage 54 or more. If they die, no labelled cells will be remaining at Stage 54 or more. Inset shows short term projection pattern at Stage 54 or more.

the changeover point then only those of the labelled cells which conform to the adult pattern will survive until the time of examination.

Since relocation and branch degeneration entail survival of cells with initially inappropriately sited axons or branches, they can clearly be differentiated from the third mechanism involving cell death.

Preliminary experiments were carried out to establish that HRP remained in the cytoplasm of the cell bodies for a sufficient length of time to enable the above experiments to be performed. Four animals ranging in age from Stage 51 to 55 were given large injections involving both the ALT and the PMT of the left limb. They were allowed to continue normal growth and development for thirteen days which is sufficient time for fast growing animals to develop from Stage 51 to Stage 54 or more. After this time they were killed and processed in the usual way.

The two main experiments were carried out on fast growing animals, injections being given at Stage 51. Great care was taken to see that no injections were given to Stage 52 animals. All the limb buds were not greater than 0.9 mm long and none showed any sign of ankle indentation. Different batches of animals were used for each experiment.

One group of animals received injections into the left PMT at Stage 51 and into the right PMT twenty hours before killing. At fixation all this group were at late Stage 54 (Table 8.1). The interval from injection to fixation was nine days.

The other group of animals received injections into

	Body length (mm)	Leg length (mm)	Stage
T 623	30	1.8	54
T 624	33	1.9	54
T 676	38	2.3	54
T 677	38	2.2	54
T 678	38	2.4	54
T 679	37	2.1	54

Table 8.1

Body lengths, leg lengths and stage of the Stage 54 delay PMT animals as measured at the time of fixation.

	Body length (mm)	Leg length (mm)	Stage
T 715	56	4.2	55
T 716	56	4.7	56
T 717	56	5.0	56
T 718	55	4.5	55
T 719	53	4.5	55
T 720	57	4.8	56

Table 8.2

Body lengths, leg lengths and stage of the delay ALT animals at fixation.

the left ALT and the right PMT both at Stage 51. At fixation all animals of this group had entered Stage 56 (Table 8.2). The interval from injection to fixation was ten days.

8.2 Nomenclature

The following nomenclature will be used in describing and discussing the results.

"Delay ALT" refers to the experiment where injections were given into the Stage 51 ALT and the animal fixed for examination at Stage 56.

"Stage 54 delay PMT" and "Stage 56 delay PMT" refer to those experiments where injections were given into the PMT at Stage 51 and the animals fixed for examination at Stage 54 and 56 respectively.

"Short-term" indicates experiments involving injections into the specified region either 20 or 44 hours before fixing for examination as specified.

8.3 Results

8.3.1 Control

In all four animals used in the preliminary tests, labelled cells of normal appearance both in morphology and the appearance of the label were found in moderate numbers in the left ventral horn only.

8.3.2 Delay PMT results

All Stage 54 and five out of six Stage 56 animals showed many labelled cells. One Stage 56 animal had only

two labelled cells.

Only averaged percentage distribution grids are given (Fig. 8.3). Distribution variation was no more than for short term PMT patterns of equivalent stage. Two experimental (delay side) grids are shown, one each for Stage 54 and Stage 56, one control (short term side) grid for Stage 54 and one grid for the short term PMT pattern nearest in Stage to Stage 56 (Stage 55). This is taken from Figure 7.2.

There were no major differences between experimental and control sides of the Stage 54 animals. In neither case were there significant numbers of labelled cells in the caudal fifth of the ventral horn and this conforms with the Stage 54 PMT pattern described in the previous chapter (Fig. 7.2A). Similarly, in the Stage 56 delay PMTs an insignificant number of cells in the caudal fifth of the cord were labelled.

However, certain differences from short-term equivalent stage patterns were found in both delay groups. Firstly, although the caudal end of the ventral horn contained an insignificant number of labelled cells in both cases, the centre of the distribution was more caudal in both cases than in the short term equivalent stage patterns. This was particularly noticeable in the case of the Stage 56 group. Secondly, the distribution of cells between the medial and middle parts of the ventral horn was found to differ between delayed and short-term equivalent stage patterns. Delayed animals had proportionately fewer medial cells.

Fig. 8.3

Averaged percentage distribution grids of:-

A: The delay side of the Stage 54 delay PMT group of animals.

B: The short term side (control) of the Stage 54 delay group of animals.

C: The delay PMT side of the Stage 56 group of animals.

D: The short term PMT patterns of a group of Stage 55 animals (nearest short term equivalent stage) (from Fig. 7.2 A).

Each delay grid (A and C) should be compared with its companion short term grid (B and D).
Note the absence of caudal labelled cells in both delay grids.

Stage 54

N=6
x=84
S.E.=29.4

(A) Delay

	2		2
	8	1	9
	11	7	18
1	12	5	18
1	14	3	18
1	11	5	17
	6	5	11
	3	2	5
			.
			.
3	67	28	

N=6
x=188
S.E.=58.5

(B) Control

	2	2	4
	5	4	9
	10	14	24
	12	11	23
1	7	8	16
1	4	3	8
1	4	3	8
	2	1	3
			.
			.
3	46	46	

Stage 56

N=5
x=93
S.E.=46.4

(C) Delay

			.
	2	1	3
1	10	1	12
	20	4	24
	12	3	15
1	15	5	21
	9	4	13
	6	2	8
	1		1
			.
2	75	20	

N=4
x=183
S.E.=47.9

(D) Nearest Short-term Equivalent Stage (55)

	5	3	8
	13	6	19
2	14	11	27
1	13	11	25
1	5	4	10
	3	3	6
	1	2	3
			.
			.
			.
4	54	40	

8.3.3 Delay ALT results

Of the six animals examined, five showed no labelled ventral horn cells in the left ventral horn, the side in which injections had been given into the ALT at Stage 51. In the sixth animal, twelve cells were labelled which is an insufficient number from which to obtain a reliable pattern. All the cells lay either medially or in the middle of the ventral horn and none were found at the extreme caudal end.

In all but one animal, large numbers of labelled cells were found in the right ventral horn, the side in which injections had been given into the PMT at Stage 51. These acted as controls to the delay ALT injections showing that label could still be present after the delay. They were mapped and are presented in Fig. 8.3 as the Stage 56 delay PMT grid.

A further feature was found in both delay PMT and ALT animals. Some brown staining debris was seen in the ventral horns on the sides corresponding to the delay experiments, that is, the left ventral horn of the Stage 54 group and both ventral horns of the Stage 56 group. This was particularly prominent in the former.

The debris consisted mainly of clusters of roughly shaped different sized globules (Fig. 3.2 B) which bore a strong resemblance to the end products of ventral horn cell degeneration (Hughes, 1961).

No attempt was made to map the distribution of the debris though it was visible to a variable extent in most areas of all delay side ventral horns including the caudal

region of the 54 delay PMT. Little debris was visible in the 56 delay animals but both left and right ventral horns contained some and on the right its distribution included the caudal region.

8.4 Discussion

8.4.1 Interpretation of results as showing cell death

The results show that cells which were inappropriate to the adult pattern and which were labelled at Stage 51 are no longer visible after a three to five stage delay. Cells which were appropriate, however, were still visible. Before concluding that therefore the inappropriate cells have died, other possible causes for the failure of labelled cells to be seen in the inappropriate regions after the delay will be considered. Firstly, is it possible that these cells have metabolised the HRP during the delay rendering them invisible although they are still alive? This is thought to be unlikely since this would imply that inappropriate cells deal with HRP in a different manner or at a different rate than the cells which are still labelled after the delay. In the case of cells innervating the PMT, such a difference cannot arise from the difference in rostro caudal position since rostral labelled cells also cease to be visible after a delay when the ALT is injected at Stage 51 but not when the PMT is injected at Stage 51 in the same animal. It is assumed that it is unlikely that cells dispose of HRP sooner by virtue of relocating their endings or because some of their axonal branches degenerate. Since these are the only reasonable differences that these cells

might have from cells remaining labelled, on the assumption that they are still alive, then differential disposal of HRP is considered a remote explanation. In addition, the presence of the brown staining debris which was noted at the caudal end of the delay PMT ventral horns and within delay ALT ventral horns supports the idea that degeneration of labelled cells has taken place in these regions.

A more serious objection is that all cells might dispose of HRP quickly but go on taking it up so long as they have endings in the region of the injection. Previous evidence (Zacks and Saito, 1969; Turner and Harris, 1974) suggests that HRP does not remain available for uptake for more than a few hours in other tissues including adult rat intercostal muscle. However, the evidence of the present experiments themselves also rules out this objection. If HRP remained available for uptake for more than a day or two, then in the case of the ALT injections, later generated ventral horn cells innervating the ALT would become labelled and be visible on examination after the delay. Such labelled cells would be expected to be found within the boundaries of the region of the ventral horn projecting to the ALT in the adult. No ventral horn cells conforming to this pattern were ever seen. Since ventral horn cells appropriate for the ALT probably begin to be laid down in Stage 52 then HRP must remain available for less than one stage. In the Stage 56 group of animals, the average stage duration was two days. It is therefore reasonable to conclude that HRP is available for uptake for less than two days. If HRP

is available for less than two days and it can still be visible within the cell cytoplasm at least thirteen days after injection, cells must take at least eleven days to dispose of it.

The possibility exists that the reason why five animals of the delay ALT group had no labelled cells after the delay was that no cells had taken up material in the first place. Of seventeen Stage 51 animals with injections confined to the ALT and examined 44 hours later, fourteen had labelled cells and three, none (Table 5.2). That is, the probability of an injection into the ALT at Stage 51 not resulting in labelled cells is approximately 0.2. Assuming the same procedure and probability for the present series of Stage 51 injections, and assuming that the animal with labelled cells received an injection that was confined to the ALT then the probability of five out of six animals having no labelled cells is

$$[p\ (\text{no cells})\ ]^5 \times [p\ (\text{cells})\ ]^1 \times \text{total number of animals}$$

$$= (0.2)^5 \times (0.8)^1 \times 6$$

$$= 0.0015$$

It can therefore be accepted from this very low probability that cells did become labelled but disappeared during the delay.

The labelled cells found in the single animal of the six delay ALTs do not conform to the normal adult ALT pattern. Instead they appear to conform to the pattern found on the right side of the same group of animals, that is the side which received PMT injections at Stage 51. Of

the twenty-one Stage 51 animals which were given injections into the ALT described in Chapter 7, four were found, on subsequent histological examination, to have involvement of both the ALT and the PMT by the injection. It is therefore likely that of the six ALT injections given at Stage 51 in the present series, one could have involved the PMT. The nature of the experiment prevents histological verification of this assumption. However, it seems likely that the labelled cells described in the single case could have resulted from involvement of the PMT. They fulfil the resulting expectation of falling into the pattern found for the delay PMT experiment.

The absence of caudal labelled ventral horn cells in the delay PMT could conceivably be the result of further caudal elongation of the ventral horn after the injections were given, that is from Stage 52 onwards, by the laying down of new ventral horn cells. Proof that this is not the case is two fold.

1. The autoradiographic study cited earlier (Prestige, 1973) shows that the whole length of the ventral horn is established by Stage 51. No new caudal additions were seen after this and particularly not at Stage 52 or more.

2. The emergence points of the dorsal roots remain constant throughout development (Prestige, 1970) thus providing markers against which to measure the caudal extent of the ventral horn. No increase in caudal extent as measured by this criterion is visible after Stage 51.

It is concluded that all the cells that were innervating the ALT at Stage 51 and the caudal cells of the Stage 51 PMT

patterns have died. The significance of this result in relation to current knowledge on cell death will be discussed in the concluding chapter. However, it should be emphasised that the cells deaths shown here have not been shown to account for all cell deaths. Other causes or roles for cell death may also exist.

8.4.2 Homology of injection sites

Although the most caudal fifth has no significantly greater number of labelled cells, the distribution of the delay PMT patterns shows a more caudal bias than the short term equivalent stage patterns. At Stage 51, though the foot is not yet determined, some below knee areas are (Tschumi, 1957). It is probably not possible with the present technique to avoid encroachment of the injection site in the Stage 51 PMT on the proximal part of the already determined plantaris region. The average percentage distribution pattern derived from four Stage 59 animals receiving injections into plantaris is shown in Fig. 8.4. It can be seen that if the Stage 51 injections did encroach on the prospective plantaris some more caudal neurones innervating the plantaris would have become labelled. Since some of these neurones might be expected to have conformed to the adult pattern for plantaris, they would have survived the delay-period and introduced a caudal bias in the delay patterns. In short-term equivalent stage studies, there is no danger of the plantaris being involved by the injection. It is considered that this is a sufficient explanation for the caudal bias and it is not necessary to invoke the

N=4
x=123

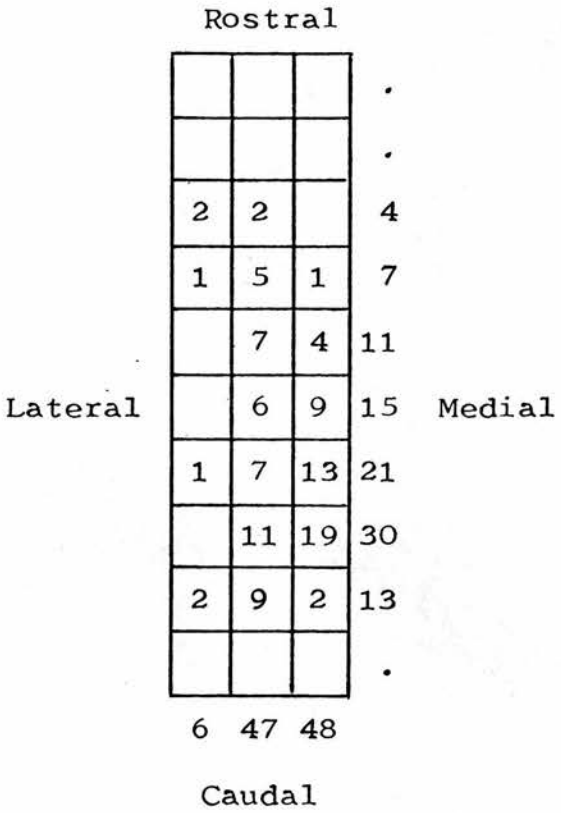


Fig. 8.4

Averaged percentage distribution grid of labelled cell distributions resulting from injection into the plantaris muscle of juveniles.

possibility that some ventral horn cells less inappropriate to the adult pattern are able to relocate their axons or undergo branch degeneration. However, it should be emphasised that either of these mechanisms may be operating at a level below the resolution of the technique, for example, within individual muscles and hence be escaping detection.

The delay PMT patterns show a smaller proportion of medial cells than the short term equivalent-stage patterns. There is no ready explanation for this. It is possible that a similar mechanism is at work here to that responsible for the caudal bias. In Stage 51 animals PMT injections tend to involve the whole compartment, both superficial and deep whereas in older animals injections are generally more superficial. If the most superficial muscles of the PMT are represented most medially in the ventral horn then the difference is readily explicable using the same reasoning as that used to explain caudal bias.

Apart from the minor differences noted above, the pattern for delay 54 and 56 PMTs are almost the same as for short term equivalent stage patterns, that is the patterns are almost homologuus. This can be used as evidence that the region called the PMT at Stage 51 will become the PMT at Stage 54 or 56 and that homology of injection sites is being maintained in its major aspects. Since at early limb bud stages, the ALT and the PMT are, in terms of the present technique, opposite halves of the proximal limb bud (since smaller regions are below the resolution of the injection technique), then if the PMT is remaining homologous during

development, so must the ALT. This means that neither rotation, nor major rearrangement by differential growth alters the relative positions of the limb bud regions during development. This argument rests on the assumption that the ALT and PMT sites at Stage 51 are separate. Proof that they are derives from the results of this chapter. The fact that no cells innervating the ALT at Stage 51 survive to Stage 56 proves that ALT injections cannot be involving the PMT since in that case some cells would survive. This also shows that HRP cannot diffuse from the ALT to the PMT, a distance of not more than 200 μm.

8.4.3 Conclusion

The results of this chapter show that the major changes of innervation pattern to the two thigh regions which take place during Stage 53 are due to death of cells innervating regions inappropriate to the adult patterns. In the case of the ALT a complete replacement of the original innervation takes place while for the PMT, many if not all of the cells innervating it in the stages after the change-over belonged to the original set of cells innervating it. It is likely that some of the earliest cells innervating the PMT continue to innervate it in the adult though this has not been proved.

CHAPTER 9

DISCUSSION AND SYNTHESIS

9.1 Introduction

The results of Chapters 5, 7 and 8 form a coherent picture of the way in which the motor innervation of the limb develops. The limb bud is first innervated at Stage 50 by the more medial cells of the ventral horn which itself is only now appearing. As cells are laid down in a medio-lateral order so they innervate the limb in that order. While at Stage 50 the limiting factor in the initiation of innervation is probably the timing of the appearance of the ventral horn cells, the limiting factor in the subsequent extent of innervation in the proximo-distal axis is probably the development of the mesenchyme at the distal end, to a maturity sufficient to allow innervation, perhaps by its leaving the progress zone.

In Stages 50 and 51 before the foot and ankle regions are established, all ventral horn cells innervating the limb are obliged to innervate proximal tissue and since the entire length of the ventral horn is established by Stage 51, this means the most caudal cells are obliged to innervate areas other than those which caudal cells innervate in the adult. However, the innervation of the proximal tissues is not uniform. The proportion of cells innervating postero-medially is probably several times that innervating antero-laterally. Indeed, those caudal cells obliged to innervate proximally in Stages 50 and 51 nearly all innervate the PMT and not the ALT. More rostral ventral horn cells are much more likely to innervate the ALT though still in relatively

small numbers.

At the end of Stage 52 and during Stages 53 and 54 more cells are laid down on the lateral side of the ventral horn and give rise to a population of cells which innervate antero-lateral regions and not postero-medial regions. At the same time, the foot and ankle regions become established and innervated by cells from the same ventral horn regions as innervate the foot and ankle regions in the adult. The medial ventral horn cells innervate only postero-medially and lateral ventral horn cells only antero-laterally in these distal regions.

During Stage 53, those cells that up to Stage 51 at least innervated regions not appropriate to the adult pattern, that is the caudal cells that were obliged to innervate the PMT and all the cells innervating the ALT, degenerate. This process coincides with the onset of myotube formation in the thigh. By the end of Stage 54 the adult pattern of innervation is established, and the first spontaneous movements of the limb appear.

The function of the innervation taking place so early in limb bud development is quite unknown. Neither is there any information about interactions between the nerve fibres and the limb tissue.

The evolution of the adult pattern of innervation may be thought of initially as a two-stage process. In the first stage, fibre endings come into apposition with various muscle regions and in the second, any inaccuracies arising in the first are corrected by the death of ventral horn cells with inappropriately located endings.

9.2 First Stage: The Processes Governing Apposition

The early peroxidase detectable innervation patterns are ordered. Many more cells innervate the PMT than the ALT at early stages. The most caudal cells almost uniformly innervate the PMT and not the ALT. The antero-lateral and postero-medial distal regions are probably innervated by only lateral and medial ventral horn cells respectively from the time of the earliest distal innervation. These patterns can be interpreted as showing that some form of fibre guidance takes place as the growing fibres innervate the limb. However, as was discussed earlier (7.3.3) there is a possibility, though considered unlikely, that an earlier phase of less ordered innervation takes place which remained undetected. This may involve a phase of rapid re-location or branch degeneration. However, as was pointed out, these processes may be required to a greater or lesser extent to mediate fibre guidance no matter what guidance system is postulated and therefore the question is, in a sense, semantic.

The results of this thesis do not provide a definite answer as to which of the various proposed fibre guidance mechanisms (1.4) operates in the developing limb. However each of the mechanisms will be assessed in the light of the results.

9.2.1 Competition model (Prestige and Willshaw, 1975)

It is appropriate to discuss this model at this point although it might be considered to be primarily a theory about intercellular recognition rather than fibre guidance.

It also requires rapid relocation and/or branch degeneration in order to bring fibres and muscle cells into frequently changing apposition, itself an absolute requirement of the model. However, the result of this process is to bring the fibres into ordered apposition with the muscle cell array before they become committed to given post-synaptic sites. This can be thought of as corresponding to the decision making process postulated for fibre guidance (7.3.3). Since the result is ordered innervation as defined earlier (7.3.3), the competition model can also be thought of as a special form of fibre guidance.

According to the model, fibres become ordered according to a property (affinity) which is graded across both the pre-synaptic and post-synaptic cells. Gradients of various types exist in the major axes of the limb and the ventral horn. Temporal gradients are known to exist in the rostro-caudal (Hughes, 1968; Prestige, 1970, 1973) and medio-lateral axes of the ventral horn (Prestige, 1973) and the proximo-distal axis of the limb (Tschumi, 1957). A gradient, possibly chemical, has been found in the antero-posterior axis of the chick limb bud resulting from the action of the zone of polarizing activity (Saunders and Gasseling, 1968; Tickle et al., 1975). Similar gradients might exist in Xenopus. These gradients may act primarily in the type of process postulated by Prestige and Willshaw or secondarily by generating gradients of other properties (Wolpert, 1969, 1971).

A prediction of the Prestige and Wilshaw model is that a small number of presynaptic cells innervating a large number

of post-synaptic cells will tend towards the high end of the affinity gradient which will become filled first. If each post-synaptic cell can accept a large number of pre-synaptic endings (low saturation constraint) there will be crowding of the innervating cells in the high affinity region of the post-synaptic cell array. At early stages of limb development, about four times as many ventral horn cells innervate the PMT as the ALT. This conforms with a model of the limb in which the PMT is the high affinity and the ALT the low affinity end of the gradient and in which there is low saturation constraint. Recent unpublished observations (Prestige - personal communication) suggest that myoblasts are capable of supporting very large numbers of Phase II cells which supports the idea of low saturation constraint in the early limb bud.

The model also predicts that a certain number of lower affinity medial ventral horn cells may be forced by competition pressure to innervate the low affinity ALT at early stages if the PMT is innervated by as many fibres as it can contain (all PMT sites filled). This might account for the small number of cells innervating the ALT at early stages though it does not explain why only rostral and not caudal cells should be forced to innervate the ALT.

The early innervation patterns to the distal regions can be said to fit the model though there is no way from the results of this thesis of distinguishing between the mechanism of this model and other mechanisms to be described below. The proof or disproof of this mechanism in limb innervation begins with showing an early phase of rapid relocation or

branch degeneration by each fibre before it becomes HRP detectable.

9.2.2 Timing hypothesis

This cannot be used to explain the innervation patterns since there is no antero-posterior temporal gradient in the limb, yet the innervation pattern is most highly ordered in this axis. Neither can the modified form of timing hypothesis be invoked where the temporal gradient in the innervating neurones is matched to the relative distances of the post-synaptic cells, since antero-lateral and postero-medial limb regions are virtually equidistant from the ventral horn.

9.2.3 Chemospecific guidance

This is an attractive possibility. The theory proposes that growing endings respond to local chemical cues resulting in directed growth (Hamburger, 1962; Sperry, 1963; Attardi and Sperry, 1963) though the actual existence of such cues is speculative and their nature, if they exist, quite unknown. It is generally presumed in discussing this theory that the cues consist of graded properties in the cell matrix through which the fibres pass and that the fibres themselves possess matching graded labels and that the two sets of labels may be derived directly or indirectly from the ventral horn and limb axes discussed earlier. However this may be no more than conceptual simplification.

In its simplest form, the hypothesis proposes that each fibre grows along a path whose labels most match its own.

The pattern of limb innervation expected from this model is that the early fibres (Stages 50, 51 and early 52) being potentially medial in terms of their eventual adult position will innervate muscle normally innervated by medial ventral horn cells. At early stages, PMT is the only muscle of this type. In general, it is true that most ventral horn cells at early stages innervate the PMT. However, a small proportion of these ventral horn cells do innervate the ALT as well (Table 7.1) for which the model has no good explanation except to postulate a certain amount of inaccuracy in the system. However, this leaves unanswered the resulting question of why the early most caudal ventral horn cells should show very little inaccuracy in the antero-posterior axis since they almost entirely innervate the PMT (Figs. 7.1 and 7.2).

9.2.4 Selective fasciculation

Models based on this theory are strong possibilities. Fasciculation of fibres is a well known phenomenon in which nerve fibres preferentially grow along the surface of other fibres rather than at random through the surrounding tissues. This is assumed to result from the mechanical advantage of growing along a continuous surface. Selective fasciculation implies that fibres will grow along one fibre in preference to another. In the original theory of selective fasciculation proposed by Weiss (1941a), pathfinder fibres make connections in the periphery by an unspecified mechanism and subsequent fibres are guided to the correct end point by following either the pathfinder most like themselves or the pathfinder going to the region most appropriate

to themselves. Although Piatt (1942) showed that pathfinder fibres in the strict sense of an early special population of fibres are not required for normal innervation, the meaning of pathfinders can be interpreted simply as the first fibres to innervate the limb. While these would have to innervate either at random or by a hesitant seeking and sampling mechanism such as outlined earlier (7.3.3) later fibres would be much more efficiently directly to correct sites decreasing the chances of error. The mechanism of selective fasciculation has always been vaguely assumed to be one of varying affinity between fibres perhaps chemical in nature (Attardi and Sperry, 1963). One variation might be that information about the nature of the periphery innervated by pathfinder fibres is transported centripetally and detected and acted on by subsequently growing fibres using some process of interfibre transfer of the information.

The results of the present work could be explained by a mechanism of this nature. If fibres each individually tend to grow at random (though the presence of other fibres already innervating the limb would influence them into non-random growth) then the earliest fibres to innervate the limb will do so at random. The population of cells involved in the earliest innervation in relation to the Stage 51 ventral horn is shown in Fig. 9.1. Since all ventral horn cells at Stage 50 and 51 are potentially medial and therefore potentially innervate postero-medial muscle then according to the model, all ventral horn cells innervating after the earliest ventral horn cells have done so,

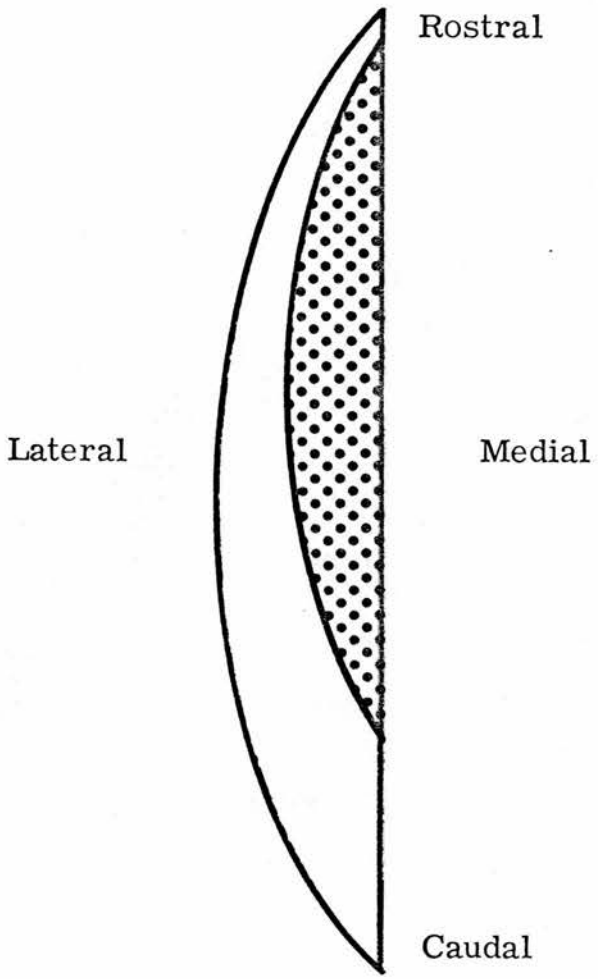


Fig. 9.1

Schematic representation of the distribution of the earliest ventral horn cells to arrive in the ventral horn (stippled area) in relation to the Stage 51 ventral horn. This diagram is inferred from the results of Prestige (1973).

should be influenced to follow those early fibres which terminate in the PMT. Since all the most caudal ventral horn cells develop after the earliest cells, they will form part of the population of cells being influenced to enter the PMT which is the only postero-medial muscle available to them at that stage. According to the model, once sufficient fibres have innervated the limb to give later coming fibres a high enough chance of having a suitable fibre to follow, no more fibres should grow into the ALT until those showing a preference for the ALT appear in about Stage 52. The result would be up to Stage 52, a pattern of cells innervating the ALT derived from the population of the earliest cells and a pattern of cells innervating the PMT derived from all cells. The patterns obtained experimentally approximately conform to this expectation (Figs. 7.1, 7.2). A further prediction of the model which is borne out by the results is that few cells should innervate the ALT compared with the PMT (Table 7.1).

The innervation of the distal regions examined in this thesis is via the sciatic nerve. The sciatic nerve divides in the proximal part of the thigh into its two main branches supplying the plantar-flexor musculature (postero-medial muscle) and the dorsi-flexor musculature (antero-lateral musculature). This division is first visible in Stage 51 as a gathering of the fascicles composing the sciatic nerve into two loose and diverging bundles. By late Stage 51 just prior to the first innervation of the foot and ankle regions, the two divisions are clearly defined. They are directed towards the postero-medial (posterior

division) and antero-lateral (anterior division) sides of the central core of denser mesenchyme destined to become the cartilaginous elements (personal observations). These observations are in agreement with those of Taylor (1943) on R. pipiens. The selective fasciculation model predicts that fibres have already been guided into one or other division before reaching the two distal regions under study resulting in a totally "correct" innervation pattern to these regions. This is seen in the present work. Very little information need be transported proximally by the pathfinders to enable succeeding fibres to choose the correct division. Information concerning the antero-posterior position of the pathfinder ending might be sufficient. There is therefore no theoretical objection in this model to fibres choosing a division before the distal regions are formed. However, it should be emphasised that the results for distal regions can also be explained by both chemospecific guidance and the competition hypothesis.

The Stage 50 ALT and PMT patterns show differences which seem at first to contradict the selective fasciculation model outlined above. The rostral fifth of the Stage 50 PMT pattern should contain more labelled cells to conform with the idea of random ingrowth by the earliest fibres. However an examination of the anatomical arrangement of the spinal nerves in relation to the base of the early Stage 50 limb bud shows that they are lined up along the antero-posterior axis of the base. They often have no interconnections and can be quite separate. Later in Stage 50 it is often seen that the eighth spinal nerve innervates only the ALT and the

tenth only the PMT while the ninth may innervate either and usually both (personal observations). These findings may be interpreted as each spinal nerve simply innervating the nearest limb tissue in an unguided fashion. Assuming that spinal nerves carry the third of ventral horn cell fibres nearest the corresponding ventral root outlet, then if the PMT receives little or no innervation from the eighth spinal nerve, it will receive correspondingly little or no innervation from the most rostral ventral horn cells. Later, fibre guidance will permit the crossing of fibres to reach the correct areas giving rise to the early plexus formation which is seen at the base of the limb at Stage 51.

An alternative explanation for the rostral differences might arise from unavoidable error in the mapping procedure. Although this might account for a small difference, a difference of this magnitude cannot be explained in this way. Errors in identifying the rostral end of the ventral horn at Stage 50 are not more than about 5%. The mapping accuracy in the rostro-caudal axis is felt to be very accurate, though mapping in the medio-lateral axis probably suffers from error of perhaps $\pm$ 10 - 15% between adjacent compartments. However an average rostro-caudal mapping error of about 20% which would be needed to explain the rostral differences is not conceivable.

9.2.5 Conclusion

In conclusion, the results most favour a mechanism which combines an initial unguided ingrowth of the earliest fibres with subsequent guidance by these of later coming

fibres. This latter process has been termed selective fasciculation after Weiss (1941a). The apparent order amongst the earliest fibres may simply be the fortuitous result of the arrangement of the early spinal nerves in relation to the limb bud.

However, alternative mechanisms, though not able to explain certain details of the results are still worthwhile possibilities. These are the chemospecific guidance model and the competition model. The latter is less likely in view of the lack of evidence for relocation or branch degeneration though it is possible that these could have escaped detection.

9.3 Second Stage: The Correction of Appositions Inappropriate to the Adult Patterns, by Cell Death

The results of Chapter 8 show that cells innervating inappropriate regions at Stage 51 die during Stage 53. Although it is tempting to generalise that all inappropriately innervating cells die, it is not possible to be certain that some degree of branch degeneration or fibre relocation does not take place over distances below the resolution of the technique within perhaps individual compartments. In fact it seems likely that branch degeneration probably does occur at this level (see below). However, as far as the major pattern changes are involved, cell death is the mechanism.

9.3.1 The relationship between cell death and muscle differentiation

The two regions in which major pattern changes occur are both thigh regions. During Stage 53, myotube formation takes place in the thigh (Newth, 1967). Although coincidence can only suggest a relationship it seems likely that there is a causal relationship between myotube formation and cell death, though a common cause of these two events which may be otherwise unrelated must be kept in mind. An example might be the rise in thyroxin level.

Post Phase I cells require contact with limb bud tissue in order to survive (Prestige, 1967, 1970). That is limb bud tissue has some property which prevents them dying. Prestige has called this maintenance factor without specifying its nature though he has suggested it might be an actual substance transported to the cell body. However, it could be no more than information, such as that required to induce afferent input to the ventral horn cell (Hendry - personal communication). In this discussion, the term maintenance factor denotes only some component of the interaction between ventral horn cells and muscle cells. Myoblasts have a maintaining property since there are Phase II cells being kept alive in late Stage 52 before myotube formation begins. Also Prestige (personal communication) has found that myoblasts of regenerating limbs are capable of supporting very large numbers of post Phase I ventral horn cells in terms of cells per limb volume compared to the normal animal. However there is the reservation that the regeneration myoblasts had stemmed from tissue that had

already been innervated. This may give them different properties from normal myoblasts (Yntema, 1959) though normal myoblasts are also innervated prior to the appearance of Phase II cells.

With the onset of myotube formation, fundamental changes occur in the muscle cell membranes. Firstly there are the changes occurring during fusion some of which are required for fusion (Stockdale and Holtzer, 1961; Okazaki and Holtzer, 1966; Bischoff and Holtzer, 1970; Easton and Reich, 1972) and secondly there are changes resulting from the formation of neuromuscular junctions (NMJ). The region of membrane outside the end plate region takes on different properties from its pre-innervation state. Its acetylcholine sensitivity disappears (Diamond and Miledi, 1962; Kano and Shimada, 1971) and it becomes refractory to further NMJ formation (Bennett and Pettigrew, 1974a,b). Only the end plate region possesses the ability to support NMJs. That the NMJ mediates the transfer of maintenance factor from the post innervation muscle cells is supported by finding that botulinum toxin interferes with nerve-muscle trophic interactions in mammals (Thesleff, 1960; Watson, 1969). Prior to myotube formation, that is before NMJs can form, the transfer of the maintenance factor must be taking place across the myoblast cell membrane in which no regional differences have so far been shown.

It seems a likely possibility that prior to the membrane changes occurring with fusion and NMJ formation, the whole muscle cell membrane is capable of supporting post

Phase I cells. With the membrane changes, the area of membrane capable of this function becomes constricted to the zone at which the NMJ will form, or to the zone of the NMJ during its formation. Any post Phase I cells unable to form a junction at this region will die. Although the evidence from other species and in vitro suggests that initially the end plate region is innervated by more than one motoneurone (Shimada et al., 1969; Redfern, 1970; Bagust et al., 1973; Letinsky, 1974), it is possible that this is insufficient to accommodate all motoneurones in the much reduced area of membrane capable of maintenance function. This would mean that a certain number of neurones would die during myotube formation.

9.3.2 Selective cell death

The scheme outlined above gives a relatively passive role to the nerve fibres and does not easily explain why there should be selective cell death. An additional idea is that all fibres attempt to synapse at random on the cell membrane but a process of interaction between the endings perhaps mediated by the muscle cell membrane leads to junction formation by only the most appropriate fibres (of which there may initially be more than one branching to the same successful end plate site). The process of junction formation by appropriate fibres aborts junction formation by less appropriate fibres and the onset of refractoriness in the extrajunctional membrane leads to death of the most inappropriate cells. An analogy with this is found in the regeneration of motor nerves to denervated muscles in fish

and tailed amphibians. Muscle fibres in these animals are normally polyneuronally innervated thus the situation resembles that occurring at the myotube formation stage in Xenopus limb muscle. Denervated muscle was experimentally reinnervated by a foreign nerve. When the original nerve was then allowed to reinnervate the muscle the foreign synapses became non-functional (Marrotte and Mark, 1970a,b; Mark and Marrotte, 1972; Mark et al., 1972; Cass et al., 1973). The foreign synapses were still present but showed a block of neuro-muscular transmission (Mark, 1975). Unfortunately there is no information about the possibility of a similar suppression of the trophic activity of the suppressed NMJs which is needed to support the idea.

This proposal predicts that in the absence of other nerve fibres, a single nerve fibre may form a neuromuscular junction on any cell regardless of the muscle cell region (Type II mechanism).

It is also necessary to consider another type of mechanism (Type I mechanism). Can a single ventral horn cell fibre form an NMJ in only one muscle region in spite of the absence of other fibres? Conceptually, a mechanism of this type is simpler. It implies a recognition system based on absolute labels and could belong to the category of the chemospecificity hypotheses (Sperry, 1943, 1944, 1945, 1963; Meyer and Sperry, 1973). It requires no more than that non-matching fibres and muscle cells are unable to form a junction due to non-recognition. These will die when the muscle cell membrane is altered cutting off the maintenance function when another, appropriate fibre forms an

NMJ. It is interesting to consider what should happen with a single fibre behaving according to a Type I mechanism where it apposes to a wrong region. It may be that post Phase I cells reach a point where an unspecialized ending is not able to mediate the transfer of sufficient maintenance factor to permit survival. The single fibre will die because it cannot form an NMJ and in spite of the muscle membrane remaining unaltered. Alternatively the ventral horn cell may survive but be unable to develop further, or else it may develop fully. The last possibility is unlikely. In either of the latter cases however, the absence of an NMJ will result in the absence of impulse transmission. In addition, nerve-muscle trophic activity is reciprocal (Guth, 1968). In the absence of innervation through an NMJ it is likely that the muscle cell will fail to develop. The likely outcome therefore of the single fibre example behaving according to a Type I mechanism and where it has wrongly apposed, is that both the nerve and muscle fibre will fail to develop and that function will certainly not develop.

The different predicted outcomes of the single fibre example behaving according to Type I or Type II mechanisms enable an experiment to be set up in principle, to distinguish which type of mechanism is operating in developing limb innervation. It is only necessary to remove or prevent the formation of a whole experimentally distinguishable region of the ventral horn such as all ventral horn cells developing after Stage 51 for example. A situation resembling the single fibre example would now

exist in the ALT and by examining this region for development and function it might be possible to make the distinction between the two types of mechanisms

Some previous experiments have given rise to situations resembling this type of experimental set up, though only little information can be gained from them. A Type I mechanism appears to operate when muscle is innervated by very foreign motoneurones during development as when axial or cranial motoneurones innervate limb muscle This is invariably followed by non-function in all species studied including Ambystoma (Piatt, 1940, 1956, 1957; Szekely, 1963), eleutherodactylus (Hughes, 1964a) and chick (Szekely and Szentagothai, 1962; Straznicky, 1963, 1967) and in the case of eleutherodactylus and chick, it is also followed by muscle atrophy. The possibility of a Type I mechanism is also suggested by two experiments in ambystoma in which motoneurones which normally innervate distal musculature appeared to be unable to innervate proximal tissue in spite of the absence of the normal source of innervation for proximal muscle (Detwiler, 1936; Straznicky and Szekely, 1967). However, in the more recent study, it is possible that innervation by thoracic axial motoneurones occurred, interfering with innervation by the limb motoneurones.

Evidence against Type I mechanisms comes from the many experiments which show that adult denervated muscle can be reinnervated by a foreign nerve resulting in fully functional NMJs in many species from fish (e.g. Sperry and Arora, 1965; Mark, 1965; Mark, 1975) to mammals (e.g. Ellsberg, 1917; Sperry, 1941, 1942; Weiss and Hoag, 1946;

Frank et al., 1975) as well as the experiments of Mark and his collaborators cited earlier which support a Type II mechanism. However, these studies are all regeneration studies on adults in which the same rules as were used in development may no longer apply. This is possible since even axial and cranial motoneurones can form functioning NMJs in adult regeneration studies (Hibbard, 1965b; Szekely and Czéh, 1971). There is no convincing evidence to support a Type II mechanism in development. The interchange of brachial and lumbar ventral horn cells which can give functioning permanent NMJs (Piatt, 1956, 1957; Szekely, 1963; Straznicky, 1963; Hughes, 1962, 1964a,b, 1968) may not be a sufficient test if the sets of labels are identical in fore and hind limbs.

Further studies are needed in this field with the specific aim of distinguishing between the two types of mechanisms in development.

9.3.3 Do all cell deaths result from inappropriate location?

As was pointed out earlier the cells which were shown to die after having made inappropriate appositions at Stage 51 are unlikely to account for all cell deaths. In fact they probably account for only a minority of cell deaths since they occur in Stage 53. The majority of cell deaths occur after Stage 53 (Prestige, 1970). Many of the latter might be explained by the death of inappropriately apposed cells in the below knee areas as this area approaches the myotube stage later than in the thigh (Newth, 1967). It is quite likely that the process of cell death shown to occur in

grossly inappropriately apposed cells also occurs for lesser degrees of inappropriate apposition and perhaps even very slight degrees. If so the single process of death of inappropriately located cells could easily account for all cell deaths. This expression of inappropriateness may not occur simultaneously with all degrees of inappropriateness but successively later with less inappropriately apposed cells. The decision about which axons should not innervate or should cease to innervate a post synaptic site is likely to be a continuation of the process which occurred for the early cell deaths, either as a Type I or Type II selection mechanism if, in the former, partial NMJ formation can occur. Thus with a Type II mechanism, competition becomes keener with development as successively less and less inappropriate endings are forced to vacate the post synaptic site eventually leaving a single vector for each site. With a Type I mechanism, only the perfectly matching ending can form a mature NMJ while less perfectly matched endings form successively less mature or efficient NMJs leading to eventual degeneration of the endings. The process of reduction of innervation from polyneuronal to mononeural however probably continues after the cessation of cell death. This can be deduced from the fact that in the rat diaphragm mononeuronal innervation does not fully result until 16 to 18 days after birth (Redfern, 1970) whereas the cervical ventral root fibre numbers cease falling by 2 days after birth (Fraher, 1974). During early stages of motor innervation in rat and chick muscle, profuse preterminal branching was seen with individual axons innervating many sites (Bennett and

Pettigrew, 1974a). Degeneration of endings may therefore lead either to cell death or not depending on the number of viable endings that a cell is left with. In the final stages of the development of mononeuronal innervation when overlapping innervation is becoming small, ending degeneration is unlikely to result in cell death. However there is no need to postulate that endings of cells that will survive behave differently to those of cells that will die. That is, the same process of ending selection occurring from myotube formation to the final mononeuronal innervation can result in early death of the most inappropriately apposed cells or successively later death of less inappropriately apposed cells or merely degeneration of a few preterminal branches without death amongst the most appropriately apposed cells.

9.4 The Relative Importance of the Two Stages of Innervation

The two stages of innervation may act independently though it is possible that both stages may make use of the same position dependent markers which might be specific molecules in the limb tissue. According to the concept of innervation which has been developed in this thesis, the two stages are complementary though partially redundant. The first stage can be thought of as bringing a selection of reasonably suitable fibres into apposition with a muscle cell and the second as choosing the most suitable of these. However, each stage acting alone could probably give an approximation to normal innervation. It is worth predicting the sorts of abnormalities that might be expected if each stage were to act alone. This would provide a basis for

further investigation and by correlating the predicted abnormalities with various clinical diseases, may enable a more accurate search for the anatomical and biochemical sites of the abnormalities responsible for these diseases.

The first stage acting alone would lead to retention of inappropriate connections which would probably result in impaired limb coordination, or spasm. For example, both the knee flexors and extensors would contract simultaneously during attempted knee flexion. Ventral horn cell numbers would be raised and there would be a normal muscle mass.

The second stage acting alone and presumably having to choose from a very disordered range of apposed fibres might show two different types of results depending on whether it operates by a Type I or Type II mechanism. A Type I mechanism would choose only those fibres which had correctly apposed. These might be expected to be greatly reduced in number as a result of not having been guided earlier. The result would probably be very large motor units and possibly some weakness and poor muscle development if the few correctly apposed fibres were insufficient to support a normal muscle complement. Also, the ventral horn cell number would be reduced. However, within the limits of any reduced muscle power, coordination would be normal.

With a Type II mechanism, because in this, ventral horn cells can innervate anywhere, there would be a normal number of ventral horn cells and motor units, and muscle mass would be normal. However, coordination may or may not be normal depending on the mechanism which controls the input to the ventral horn cells (see below). If this were unable

to change in concert with the altered peripheral connections of the ventral horn cells, then incoordination would result. Otherwise, coordination would be normal. In the latter case, the animal would appear quite normal. The only abnormality which might or might not be detectable would be in the ventral horn cell projection patterns. If such complete adaptation as this could occur then it might be asked what purpose is there in having any sort of ordered innervation at all? The existence of mechanisms to control fibre apposition and eliminate non-conforming cells is itself highly suggestive that these are essential and that complete adaptation to abnormalities of the two stages is not possible. However, this is not to say that a Type II mechanism and some adaptation is not possible.

9.5 How are Ventral Horn Cells Innervated by Appropriate Afferents?

The almost complete overlap of the projections of the lateral of the ventral horn cells to the different antero-lateral limb regions leads to the question as to how ventral horn cells become innervated by the appropriate afferent connections. The same question also arises from the overlap regions of the medial patterns. The lateral of the ventral horn cells cannot be dealt with as one homogeneous population by afferent fibres since antero-lateral muscles contract at different times during, for example, the normal swimming cycle. There are at least three types of possible answer to this question. They will be discussed in turn.

(1) The first is based on the modulation theories (Weiss, 1936, 1942; Kollros, 1943; Sperry, 1941, 1950; Jacobson and Baker, 1968, 1969). Each motoneurone takes on properties characteristic of the muscle to which it is connected and afferent fibre input is controlled by these properties. However, there is no convincing evidence that such a process occurs in neuromuscular systems though no studies have been made during development. Myotypic modulation was proposed by Weiss (1937) to explain the homologous response in experiments with axolotls after his resonance principle had been disproved (Wiersma, 1931). This was modified by Sperry (1950) to the modern theory of myotypic respecification of afferent connections to explain experiments in teleost fish which showed recovery of normal function after nerve crosses in the fins. The suspicions of Sperry and Deupree (1956) that good functional recovery will only follow reinnervation by a muscle's native nerve were confirmed independently by Mark (1965) and by Sperry and Arora (1965). They found that following nerve crosses to opposing muscles in cichlid fish with care to prevent fibres growing back to their normal muscle, only abnormal movements resulted after regeneration unless the native nerve somehow managed to reinnervate its own muscle. A further recent study also showed that normal function following nerve crosses in axolotls resulted from fibres growing back to their original muscle without necessarily being visible (Grimm, 1971). Though this all constitutes strong indirect evidence against myotypic modulation, the possibility that it might occur in development is suggested by a series of

electrophysiological studies in kittens showing variable statistical changes in afferent input to ventral horn cells as predicted by the theory of myotypic modulation (Eccles et al., 1960, 1962a,b). However, only a few of the tests were highly statistically significant and many were not significant. However it is possible that myotypic modulation is an important phenomenon in an earlier stage of development and that the work of Eccles et al., revealed only the last stages of its operation. Until specific tests have been made during early limb development, this question remains open.

(2) The second type of answer is that there are actually two populations of ventral horn cells generated and mixed together. Subtle differences between the two such as cytochemical or dendritic growth patterns enable connections by each population with only one muscle region and one type of afferent input. This is a very rigid scheme with no allowance for error.

(3) The third type of answer is that the afferent connections which a ventral horn cell receives dictates the muscle region with which it is appropriate for that cell to connect. This might be by an induction of chemospecific properties or by some other mechanism. An attractive possibility is a mechanism based on the principle of functional verification (Weisel and Hubel, 1965,1963; Hubel and Weisel, 1965; Keating, 1968; Gaze et al., 1970; Willshaw and von der Malsburg, 1975). If a ventral horn cell receives afferent stimulation from a sensory neurone that is stimulated

by the muscle cell contraction resulting from the firing of that ventral horn cell then the connections in the circuit may be strengthened. This idea can be tested by interrupting the sensory innervation to the developing limb. Experiments involving deafferentation of limbs of developing Rana tadpoles (Weiss, 1941b)failed to produce any abnormalities of limb coordination in many animals tested. Unfortunately the stage of the animals at operation was not specified, nor were the abnormalities which occurred in the abnormally functioning limbs specified. The author concluded that these abnormalities resulted from damage to other components around or in the vertebral column. However even with normally coordinated limbs, abnormalities of innervation of the type predicted from the functional verification hypothesis could exist but these need not be detectable by gross observation of movements.

It is not possible from the results of this thesis to say which of the possible mechanisms is responsible for selective afferent innervation though there may be more than one. Functional verification would be an ideal mechanism for giving the fine-tuning in the final stages of development of mononeuronal innervation though specific experiments to demonstrate its existence in the neuromotor system have yet to be done.

9.6 Conclusion

The work of this thesis has been a continuation of the investigation of the mechanisms of limb innervation. The importance of an understanding of these mechanisms is two-fold.

Firstly it will perhaps contribute to an understanding of the general principles governing the development of the whole nervous system and secondly it is required in order to investigate the causes of many disorders of the neuro-muscular system. In particular it is hoped the line of investigation taken in this work may eventually lead to an understanding of those diseases which have the appearance of a breakdown in the nerve muscle interactions. Some of these such as arthrogryposis congenita and Werdnig Hoffman disease may result from abnormalities of the nerve-muscle selection mechanisms. An example of the importance of understanding the selection mechanisms can be seen by considering the different implications to clinical disease of Type I and Type II mechanisms. In the event of damage or other insult to the ventral horn cell population during development, a Type II mechanism has the ability to adapt whereas a Type I does not. It is therefore of great interest to distinguish between these in the developing neuromotor system.

Full understanding of the innervation mechanisms may elucidate forms of treatment for some neuromuscular disorders and even lead to the development of highly efficient prostheses using biological principles. It is hoped that the work of this thesis makes a contribution towards attaining these ends.

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Fig. 1.1

Limb bud shapes and sizes at successive stages with target points for injection sites superimposed. Stages 50 to 55 are drawn to the same scale where the Stage 50 limb is 0.4 mm long. Juvenile limb is drawn to one quarter the scale of the larval limbs. Dashed circle indicates target point on side of limb farthest from view.
ALT = antero-lateral thigh. PMT = postero-medial thigh. ALA = antero-lateral foot and ankle region (larval) or antero-lateral tarsal area (Juvenile). Plantar = postero-medial foot and ankle region (larval) or plantar compartment of foot (Juvenile). Distal = distal part of limb bud (Stages 50 and 51). Mixed injections refer to those involving both the ALA and Plantar in animals of Stage 52 or more.